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Modelling oxidative stress in human hippocampal progenitor cells: insight into the pathogenesis of depression and antidepressants action

Bakunina, Nataliia Sergeevna

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**Modelling oxidative stress in human hippocampal
progenitor cells: insight into the pathogenesis of
depression and antidepressants action**

Nataliia Bakunina

A thesis is submitted in fulfilment of the requirement for the degree of

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"Life is nothing but an electron looking for a place to rest"

Albert Szent-Györgyi

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Abstract

Recent findings suggest that oxidative stress (OS) has an important role in the pathophysiology of major depressive disorder (MDD), however the underlying molecular mechanisms are still poorly understood. In this project, I set up an experimental *in vitro* OS model, using a human hippocampal progenitor cell line HPC0A07/03C capable of neurogenesis, to study molecular mechanisms potentially involved in the pathogenesis of this disorder and to evaluate the possible antioxidative properties of compounds with antidepressant properties.

Alterations of redox homeostasis were induced by treatment of the cells with a range of concentrations of *tert*-butyl hydroperoxide (*t*-BHP); higher doses were cytotoxic and lower doses led to major changes in neurogenesis and activation of the transcription factors Nrf2 and NF- κ B in a dose-dependent manner. Potential redox-signalling pathways involved in regulating neurogenesis were studied. Inhibition of the *t*-BHP-induced ERK1/2 activation abrogated the increased differentiation of progenitor cells into mature neurons observed upon oxidative stimulus. I tested various compounds for their antioxidative properties and found that the omega-3 fatty acid eicosapentaenoic acid (EPA), as well as the conventional antioxidants *N*-acetylcysteine (NAC) and glutathione (GSH) prevented the oxidative damage inflicted by *t*-BHP, with no effects observed for sertraline, venlafaxine, ketamine or docosahexaenoic acid (DHA). Both EPA and GSH differentially regulated the expression of genes involved in the OS response, including *Keap1*, *HMOX1*, *SLC7A11* and *SLC1A1*.

Data obtained in this research project indicate that depending on the dose ROS can have detrimental effects on the viability and molecular profile of human hippocampal progenitor cells or facilitate neurobiological processes that are essential for normal

cellular functioning and adaptation of the organism to environmental stimuli. Remarkably, EPA, known to exhibit antidepressant properties, elicited favourable effects in my OS *in vitro* model and appears to be a promising compound for further research in developing novel antidepressants targeting OS.

Statement of Originality

Silvia Alboni performed the analysis of kynurenine pathway metabolites levels presented in section 3.5.2. All other experiments were conducted by Nataliia Bakunina, under the supervision of Dr. Patricia Zunszain and Prof. Carmine Pariante. The human hippocampal progenitor cells were kindly provided by Professor Jack Price, on behalf of ReNeuron.

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“Modelling oxidative stress in human hippocampal progenitor cells: insights into putative underlying mechanisms of depression”

Table of contents

1	Introduction.....	23
1.1	Major depressive disorder	23
1.1.1	Definition of depression.....	23
1.1.2	Prevalence of major depressive disorder and associated risks with the disease.....	26
1.1.3	Etiological factors of depression.....	27
1.1.3.1	Genetic vulnerability to depression	27
1.1.3.2	Environmental risk factors for depression	30
1.1.4	Neuroprogressive nature of depression: introduction of the concept ..	34
1.1.5	Monoamine deficiency theory of depression.....	38
1.1.6	Hippocampus	41
1.1.6.1	Hippocampus in depression	42
1.1.6.2	Adult hippocampal neurogenesis.....	44
1.2	Oxidative stress and molecular mechanisms relevant to depression	48
1.2.1	Terminology in redox biology	48
1.2.2	Oxidative stress and cellular responses.....	50
1.2.3	Oxidative damage	57
1.2.3.1	Lipid peroxidation.....	58
1.2.3.2	End products of lipid peroxidation	59
1.2.3.3	DNA and protein damage	60
1.2.4	Glutathione (GSH) and system x_c^- as major regulators of redox homeostasis in the brain.....	62
1.2.5	Excitatory Amino Acid Transporter	65
1.2.6	Cross talk between OS and inflammation in depression	68

1.2.6.1	Nrf2 – major antioxidant transcription factor	72
1.2.6.2	NF-κB – a redox sensitive transcription factor	77
1.2.6.3	Kynurenine pathway affected by OS	80
1.2.7	Redox signalling	84
1.3	Oxidative stress in depression.....	89
1.3.1	Oxidative stress in clinical studies of depressed patients	89
1.3.2	Association between increased OS, MDD and other factors: severity of MDD, gender differences and telomere length.....	91
1.3.3	Effects of antidepressant treatments on OS in clinical studies	94
1.3.4	Findings of molecular mechanisms of OS in MDD, coming from animal models of depression.....	96
1.3.5	Antioxidant compounds with antidepressant properties.....	98
1.3.6	Omega-3 fatty acids as potential antidepressants with antioxidative properties.....	100
1.4	Human hippocampal progenitor cells as a research tool to study the role of OS in depression and effects of antidepressant compounds	103
1.5	Aims and hypothesis of the study	105
2	Materials and Methods.....	106
2.1	Study design.....	106
2.2	Human hippocampal progenitor cells and culture	109
2.3	Experimental design	115
2.3.1	Identification of the working doses of <i>t</i> -BHP	115
2.3.2	Assays with antidepressant compounds.....	116
2.3.3	Proliferation assay.....	119
2.3.4	Differentiation assay	119

2.4	Cell viability assessment by MTS assay	123
2.5	Lipid peroxidation assessment by TBARS assay	124
2.6	Immunocytochemistry	127
2.6.1	Automated quantification of immunofluorescence.....	129
2.6.2	Representative images	134
2.7	Secreted protein quantification using ELISA method	136
2.8	Multiplex protein quantification using Luminex platform.....	137
2.9	Nrf2 transactivation assay.....	139
2.9.1	Nuclear protein extraction.....	139
2.9.2	Protein quantification.....	139
2.9.3	TransAM Nrf2 assay.....	140
2.10	Electrochemiluminescence assay using Meso Scale Discovery platform.....	143
2.10.1	Whole cell lysate extraction.....	143
2.10.2	Meso Scale Discovery assay	145
2.11	Gene expression analysis	148
2.11.1	RNA isolation	148
2.11.2	Reverse Transcription Polymerase Chain Reaction (RT-PCR).....	149
2.11.3	Quantitative Real-Time PCR (qPCR).....	150
2.12	Kynurenine pathway quantification	152
2.12.1	Sample preparation	152
2.12.2	Liquid chromatography.....	153
2.13	Statistical analysis	153
2.14	Drugs and reagents	155
3	Results	156

3.1	Oxidative stress induced by <i>t</i>-BHP causes cell damage in human progenitor hippocampal cells.....	156
3.1.1	<i>t</i> -BHP causes cell damage and cell death in a dose-dependent manner, with intensity of effects dependant on time of exposure	156
3.1.2	<i>t</i> -BHP induces lipid peroxidation in a dose-dependent manner.....	160
3.1.3	<i>t</i> -BHP treatment does not cause differences in expression of Cleaved Caspase-3.....	162
3.2	Effects of <i>t</i>-BHP on neurogenesis.....	164
3.2.1	Effects of <i>t</i> -BHP treatment on proliferation.....	164
3.2.2	Effects of <i>t</i> -BHP treatment on differentiation.....	167
3.3	Modulating effect of <i>t</i>-BHP treatment on MAP kinases.....	169
3.3.1	Inhibition of ERK1/2 abrogated the increase in MAP2-positive cells induced by <i>t</i> -BHP.....	173
3.4	Oxidative stress induced by <i>t</i>-BHP causes activation of pleiotropic transcription factors NF-κB and Nrf2	178
3.4.1	<i>t</i> -BHP induces Nrf2 nuclear translocation	178
3.4.2	<i>t</i> -BHP induces NF- κ B p65 subunit nuclear translocation.....	181
3.5	Oxidative stress induced by <i>t</i>-BHP in the human progenitor hippocampal cells: in pursuit for inflammation.....	183
3.5.1	<i>t</i> -BHP treatment does not lead to increased secretion of cytokines...	184
3.5.2	<i>t</i> -BHP treatment did not affect the kynurenine pathway	187
3.6	Testing various compounds with antidepressant action for the ability to prevent oxidative damage induced by <i>t</i>-BHP	189
3.6.1	Testing conventional antioxidants for the ability to prevent oxidative damage induced by <i>t</i> -BHP	189

3.6.2	1 hour pre-treatment and 1 hour co-treatment with antidepressants plus <i>t</i> -BHP did not prevent the reduction in cell viability caused by oxidative insult.....	194
3.6.3	24 hours pre-treatment and 1 hour co-treatment with EPA plus <i>t</i> -BHP, but not antidepressants or DHA, prevented the reduction in cell viability caused by oxidative insult of the human hippocampal progenitor cells.	197
3.7	Modulation of gene expression by <i>t</i>-BHP and EPA and GSH in the presence of oxidative insult in the human hippocampal progenitor cells.....	200
3.7.1	Modulation of antioxidative stress genes by <i>t</i> -BHP and EPA and GSH in the presence of oxidative insult in the human hippocampal progenitor cells.....	201
3.7.2	Modulation of system x_c^- and glutamate transport by <i>t</i> -BHP and EPA and GSH in the presence of oxidative insult via gene expression regulation in the human hippocampal progenitor cells.....	205
4	Discussion	207
4.1	Summary of findings	207
4.1.1	Aim 1	208
4.1.2	Aim 2	209
4.1.3	Aim 3	209
4.1.4	Aim 4	210
4.2	Damaging effects of induced OS on the human hippocampal progenitor cells.....	211
4.2.1	Establishing the model.....	211
4.2.2	Lipid peroxidation.....	216
4.2.3	Cleaved Caspase-3—independent cell death.....	218

4.3	Insights into changes in neurogenesis upon redox shifts and underlying mechanisms.....	223
4.3.1	Proliferation of the human hippocampal progenitor cells upon redox shifts in the system.....	225
4.3.2	Differentiation of the human hippocampal progenitor cells upon redox shifts in the system.....	226
4.3.3	MAPK modulation in the human hippocampal progenitor cells by <i>t</i> -BHP.....	230
4.3.4	ERK1/2 as a potential signalling pathway of <i>t</i> -BHP-induced neural maturation of the human hippocampal progenitor cells.	232
4.4	Crosstalk between redox and inflammatory systems	235
4.4.1	Activation of Nrf2 and NF-κB transcription factors with no signs of inflammation.....	235
4.4.2	Kynurenine pathway	239
4.5	Investigating the antioxidative properties of antidepressants and omega-3 fatty acids	241
4.5.1	NAC and GSH, but not antidepressants exhibited antioxidative properties.....	241
4.5.2	EPA, but not DHA, protected cells from oxidative damage.....	243
4.6	Modulation of gene expression by EPA and GSH	246
4.6.1	<i>Keap1</i> and <i>HMOX1</i>	246
4.6.2	<i>NQO1</i> and <i>GCLM</i>	249
4.6.3	Modulation of system x_c^- and glutamate transport by the compounds under investigation via gene expression	251
4.6.4	Summary on gene expression modulation by EPA and GSH.....	254

4.7	Methodological considerations	254
4.7.1	The use of an in vitro system	255
4.7.2	Cell type	257
4.7.2.1	c-Myc construct is a potential confounder of the cellular model...	257
4.7.2.2	Immune responses in this cellular model.....	259
4.7.3	Studying OS <i>in vitro</i>	260
4.8	Potential applications of the cellular model and future directions.....	262
4.9	Conclusion	264
5	References.....	270

Figures

Figure 1. A combination of factors leading to a major depressive episode	37
Figure 2. Serotonin neurotransmission.	40
Figure 3. Development of the dentate gyrus granule cells to fully mature neurons ...	47
Figure 4. General cellular responses to different levels of OS	56
Figure 5. System x_c^-	64
Figure 6. Mechanisms underlying transport of L-cystine via xCT and L-cysteine via other neutral amino acids transporters	65
Figure 7. Proposed closed loop of inflammation and ROS production	70
Figure 8. Activation of transcription factors by ROS	76
Figure 9. Canonical and non-canonical NF- κ B signalling pathways.....	79
Figure 10. Simplified kynurenine pathway of tryptophan metabolism	82
Figure 11. Schematic molecular consequences of OS that are hypothesized to take place in the pathophysiology of depression	83
Figure 12. MAPK cascades.....	88
Figure 13. Overview of experimental techniques employed in this PhD thesis	108
Figure 14. Schematic of ReNeuron's HPC0A07/03C cells with c-myc- ER TM technology.....	112
Figure 15. Experimental design	117
Figure 16. Experimental design for the cell viability assay	121
Figure 17. Assay Principle for the TBARS Parameter Kit.	126
Figure 18. An automated approach to quantifying the number of cells and markers of interest using the Cell Insight Platform (ThermoScientific).....	131
Figure 19. Example of an automated identification of the area of interest.....	132
Figure 20. Algorithm of an automated identification of the area of interest	133

Figure 21. Representative images of neurogenic markers acquired using the Cell Insight Imaging Platform (ThermoScientific)	135
Figure 22. Cytokine Human Magnetic 25-Plex Panel protocol	138
Figure 23. Flow chart of Nrf2 transactivation assay using the Nrf2 TransAM kit from Active Motif	142
Figure 24. Spot diagram showing placement of analyte capture antibody	147
Figure 25. Principle of electrochemiluminesce in an immunoassay	147
Figure 26. Dose-dependent decrease in cell viability after 24 hours of <i>t</i> -BHP treatment	157
Figure 27. Dose-dependent decrease of cell viability after 1 hour of <i>t</i> -BHP treatment followed by 23 hours of culturing in fresh media	159
Figure 28. Dose-dependent increase in lipid peroxidation after 1 hour <i>t</i> -BHP treatment	161
Figure 29. Treatment of cells with <i>t</i> -BHP for 1 hour followed by 23 hours of culturing in fresh media did not cause differences in the expression levels of Cleaved Caspase-3	163
Figure 30. Treatment of human hippocampal progenitor cells with <i>t</i> -BHP for 72 hours of proliferation caused decrease in Ki67-positive cells	166
Figure 31. Treatment of human hippocampal progenitor cells with <i>t</i> -BHP during proliferation and differentiation caused increase in neurogenesis and decrease in the number of cells	168
Figure 32. <i>t</i> -BHP treatment during proliferation and differentiation did not cause changes in the levels of phosphorylated MAP kinases	170
Figure 33. <i>t</i> -BHP treatment for 24 hours during proliferation did not cause changes in the levels of phosphorylated MAP kinases	172

Figure 34. Experimental design of the assay with FR180204 – ERK1/2 inhibitor ..	175
Figure 35. <i>t</i> -BHP-induced increase of neuroblasts and the effect of FR180204 inhibitor.....	176
Figure 36. Abrogation of <i>t</i> -BHP-induced increase of neuronal maturation by blocking ERK1/2 with FR180204 inhibitor.....	177
Figure 37. Dose-dependent increase of the amount of Nrf2 in nuclear protein extracts after 1 hour of <i>t</i> -BHP treatment	180
Figure 38. <i>t</i> -BHP treatment of the human hippocampal progenitor cells caused increase in the nuclear translocation of the NF- κ B p65 subunit.....	182
Figure 39. Standard curve based on the results from the provided standards in IL-6 Quantikine ELISA kit (R&D Systems) generated using SoftMax Pro (Molecular Devices)	185
Figure 40. Metabolites of the kynurenine pathway detected in the supernatants of the human hippocampal progenitor cells treated with <i>t</i> -BHP.....	188
Figure 41. 1 hour pre-treatment and 1 hour co-treatment with 100mM of NAC plus <i>t</i> -BHP of the human hippocampal progenitor cells prevented reduction in cell viability caused by <i>t</i> -BHP	191
Figure 42. 24 hours pre-treatment and 1 hour co-treatment with GSH plus <i>t</i> -BHP followed by 23 hours of incubation in fresh media of the human hippocampal progenitor.....	193
Figure 43. 1 hour pre-treatment and 1 hour co-treatment with antidepressant compounds plus <i>t</i> -BHP of the human hippocampal progenitor cells did not prevent reduction in cell viability caused by <i>t</i> -BHP.....	195

Figure 44. 24 hours pre-treatment and 1 hour co-treatment with antidepressant compounds plus <i>t</i> -BHP followed by 23 hours of incubation in fresh media of the human hippocampal progenitor cells	199
Figure 45. Modulation of antioxidative gene expression by <i>t</i> -BHP and EPA and GSH in the presence of <i>t</i> -BHP in the human hippocampal progenitor cells	203
Figure 46. Modulation of <i>SLC7A11</i> and <i>SLC1A1</i> genes by <i>t</i> -BHP and EPA and GSH in the presence of oxidative in the human hippocampal progenitor cells.....	206
Figure 47. Release model of regulation of the Keap1–Nrf2 pathway	248

Tables

Table 1. The evidence for foetal programming of depression	33
Table 2. Family of NOX enzymes and their distribution in the central nervous system	55
Table 3. Components added to DMEM for proliferation media.	113
Table 4. Components added to DMEM for differentiation media	114
Table 5. Comounds under investigation in this thesis.....	118
Table 6. Plates and number of cells used in the experiment according to the protocol requirements of the assay	122
Table 7. List of primary antibodies	128
Table 8. Complete Lysis Buffer.	144
Table 9. qPCR reaction mix	151
Table 10. Concentrations of IL-6 in pg/ml in the supernatants collected from the human hippocampal progenitor cells treated with a range of concentrations of <i>t</i> -BHP after 1 hour	186
Table 11. Summary of the findings obtained in this thesis, proposed future research and potential implications of the results	266

List of abbreviations

3-HANA	3-hydroxyanthranilic acid
3-HK	3-hydroxykynurenine
4-HNE	4-hydroxy-2-trans-nonenal
4-OHT	4-hydroxytamoxifen
4F2hc	4F2 heavy chain
5-HT	5-hydroxytryptamine
5-HTTPR	Serotonin-transporter-linked polymorphic region
8-OHdG	8-hydroxy-2'-deoxyguanosine
AHN	Adult hippocampal neurogenesis
ACTH	Adrenocorticotrophic hormone
Akt	RAC-alpha serine/threonine-protein kinase
ALA	α -linolenic acid
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA	amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANA	Anthranilic acid
ARE	Antioxidant responsive element
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
CAT	Catalase
CC3	Cleaved caspase-3
DAMPs	Damage-associated molecular patterns
Dcx	Doublecortin
DHA	Docosahexaenoic acid
DiHCl	Dihydrochloride
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition
EAAT	Excitatory amino acid transporter
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELS	Early life stress
EPA	Eicosapentaenoic acid

ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinase
GABA	γ -Aminobutyric acid
GCL	Glutamate cysteine ligase
GCLM	Glutamate cysteine ligase modifier
GSH	Glutathione
GSSG	Glutathione disulphide
GWAS	Genome-wide association studies
HAM-D	Hamilton Depression Rating Scale
HO-1	Heme oxygenase 1
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
ICD-10	International Classification of Diseases, 10
IDO	Indolamine-2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IsoP	Isoprostane
JNK	C-Jun N-terminal kinase
KAT	Kynurenine aminotransferase
Keap1	Kelch like ECH associated protein 1
KMO	Kynurenine 3-hydroxylase
KYN	Kynurenine
KYNA	Kynurenic acid
KYNU	Kynureninase
LPS	Lipopolysaccharide
MAO	Monoamine-oxidase
MAP2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MDD	Major depressive disorder

MKKK	MAPK kinase kinases
MKP1	MAP kinase phosphatase 1
MnSOD	Manganese-superoxide dismutase
mtDNA	Mitochondrial DNA
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
NAC	<i>N</i> -acetyl-cysteine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
NMDA	<i>N</i> -methyl-D-aspartate
NOX	NADPH oxidase
NQO1	NADPH:quinone oxidoreductase 1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NRROS	Negative response ROS
NSC	Neural stem cell
OS	Oxidative stress
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3-Kinase
PUFA	Polyunsaturated fatty acid
QUINA	Quinolinic acid
rec	Recombinant
RMM	Reduced modified media
ROS	Reactive oxygen species
SERT	Serotonin transporter
<i>SLC1A1</i>	Solute carrier family 1 member 1 (Excitatory amino-acid transporter 3)
<i>SLC6A4</i>	Solute carrier family 6 member 4 (sodium-dependent serotonin transporter)
<i>SLC7A11</i>	Solute carrier family 7 member 11 (xCT)
SNRI	Serotonin-norepinephrine reuptake inhibitor
SODs	Superoxide dismutases
SOX2	SRY (sex determining region Y)-box 2

SSRI	Selective serotonin reuptake inhibitor
SVZ	Sub-ventricular zone
<i>t</i> -BHP	<i>tert</i> -butyl hydroperoxide
TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reactive substances
TDO	Tryptophan-2,3-dioxygenase;
TDO	Tryptophan-2,3-Dioxygenase
TNF- α	Tumour necrosis factor- α
TPH	Tryptophan hydroxylase
TRP	Tryptophan
WHO	World Health Organisation
xCT	Sodium-independent cystine-glutamate antiporter
3-HANA	3-hydroxyanthranilic acid
3-HK	3-hydroxykynurenine
4-HNE	4-hydroxy-2-trans-nonenal
4-OHT	4-hydroxytamoxifen
4F2hc	4F2 heavy chain

1 Introduction

In the introduction I will present major depressive disorder (MDD) in the context of the neuroprogressive concept, resembling similar clinical, structural and molecular abnormalities observed in neurodegenerative disorders. Hippocampus and adult hippocampal neurogenesis that are known to be affected in MDD are then discussed to provide a rational for using the cell line utilised in this thesis. Oxidative stress (OS), various aspects of redox biology and molecular mechanisms that have been implicated in the pathogenesis MDD are summarised, including sections on specific redox-relevant molecular aspects that have been further investigated in this project. Evidence of the involvement of OS in the pathogenesis of MDD, which is the major focus of this thesis, is critically reviewed covering clinical and animal studies. This is followed by a discussion of antidepressant and antioxidative properties of various compounds with a particular emphasis of omega-3 fatty acids, which have been examined in this project.

1.1 Major depressive disorder

1.1.1 Definition of depression

MDD is a multifactorial complex disorder characterised primarily by persistent low mood or loss of interest or pleasure in usual activities. These are core symptoms that are often accompanied by diminished ability to think or concentrate, weight changes, insomnia or hypersomnia, fatigue or loss of energy, psychomotor agitation or retardation, feelings of worthlessness or excessive or inappropriate guilt and recurrent suicidal ideation in most severe cases. The symptoms cause significant distress and

impairment in patient's everyday life and are not due to any general medical condition or physiological effects of medication or other substances. These are the diagnostic criteria of depression that are formulated in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5, 2013) published by the American Psychiatric Association and in the International Classification of Diseases, (ICD-10, 2007) published by the World Health Organisation (WHO) and used across its member states.

Depressive symptoms are normally assessed by a psychiatrist, psychologist or a general practitioner using a variety of scales including the Hamilton Depression Rating Scale (HAM-D) that has been used for the last 40 years as a golden standard (Bagby et al., 2004). Despite a huge range of instruments for identifying depression being available, there is still no biological test that can confirm the presence of this disorder. This obstacle raises a great concern about the objectification of the diagnosis. Clinicians aim at justifying the predominant symptomatology and possible biological, psychological and social factors, which may impact on a patient's mood and the course of the disease, in order to suggest the most relevant treatment option.

Notably, despite all the psychological and biological treatments being administered to patients, major challenges such as achieving only partial therapeutic response, high levels of relapses and recurrence and treatment resistant cases are to be addressed (Richards, 2011).

Additionally, the fact that the first line treatment of depression remains to be antidepressants, which is a biological treatment, and the absence of a clinically valid biological confirmation of the disorder brings up the importance of delineating molecular mechanisms of depression. Moreover, there is currently no way to determine which antidepressant will work best for a given patient and there is lack of

guidance in the medical literature for clinicians to decide which drug to try next if one does not show a desirable effect. Furthermore, the fact that in other areas of medicine the diagnostic is defined by validated biomarkers and the absence of such a biomarker in depression creates stigma around individuals suffering from depression as the society often doubts that the disorder is “real”, which also hinders patient’s recovery. The validated biomarkers are measurable ‘objective physiological indicators of normal biological processes, pathogenic processes or response to a specific therapeutic intervention’ (Colburn et al., 2001). The benefits of having and identifying an aetiologically-sound and objective panel of biomarkers for depression are significant. Biomarkers can be used to confirm the presence of the disorder, to predict probability of its onset, to indicate the disease prognosis, to classify the disorder according to severity and symptomatology, to predict response and track progress following a therapeutic intervention (Colburn et al., 2001; Gururajan et al., 2016), and finally, to combat stigma surrounding depressive disorder.

Realistically, in most patients the complex and heterogeneous nature of depression has contributions from multiple pathways that are likely to be dependent of one another; therefore, identifying a single biomarker might not be sufficient. In any case, this information gap can be addressed by research that is aimed at identifying particular molecular mechanisms thought to be involved in the pathogenesis of depression. This would help to identify potential biomarkers which could be used as a diagnostic marker of depression and antidepressant treatment outcome along with new targets for designing efficient pharmacological treatments.

1.1.2 Prevalence of major depressive disorder and associated risks with the disease

According to the WHO depression is the leading cause of disability worldwide, and is a major contributor to the global burden of disease with an estimated 350 million people affected globally (who.org). WHO projects that the disability adjusted life-years (the sum of life-years lost due to premature death and years lived with disability adjusted for severity) will be the highest for depression by 2030 (Lepine and Briley, 2011). That means dramatic economic consequences for the modern society due to decreased workplace productivity and absenteeism resulting in lowered income or unemployment. Over 75% of patients with major depression experience repeated episodes and exacerbations throughout life. More than 50% of patients who recover from a first depressive episode have a second within six months unless they are given maintenance antidepressant treatment (Gold et al., 2015). Moreover, only partial response to treatment is often achieved. The mortality risk for suicide in depressed patients is more than 20-fold greater than in the general population (Bostwick and Pankratz, 2000) and as many as 15% of patients who never receive any treatment attempt suicide (Gold et al., 2015). The problem of MDD extends far beyond the illness itself affecting not only the quality of life but also the life expectancy and general health condition of an affected individual. A great number of studies reported increased mortality and morbidity rates associated with depression (Maurya et al., 2016). In particular, evidence supports an association between both late-life as well as early-life depression and dementia (Bennett and Thomas, 2014; Byers and Yaffe, 2011; Cherbuin et al., 2015; Geerlings et al., 2008). As such, subjects with an early onset of depression showed increased risk for Alzheimer's disease (Geerlings et al., 2008), while individuals with late-life depression have been consistently associated

with a two-fold increased risk of different types of dementia, as reported by a recent meta-analysis that included a sample size of 66535 participants (Cherbuin et al., 2015). It is clear that depression presents a major challenge for society that will benefit from further research.

1.1.3 Etiological factors of depression

Risks factors for depression fall into two larger categories - genetic and environmental, either on their own or most commonly interacting with each other. Inherited risk factors include a family history of depression, female gender and certain personality traits. Genetic preconditions also encompass variants in particular genes, such as the serotonin transporter (*SLC6A4*) and brain-derived neurotrophic factor (*BDNF*) genes. Environmental determinants include early life stress or trauma, maternal depression, unhealthy lifestyle choices and stressful life events later in life (Heim and Binder, 2012). Both risks will be described in further detail.

1.1.3.1 Genetic vulnerability to depression

Genetic heritability for MDD is estimated to be around 35-40% (Jansen et al., 2015). A family history of depression is widely known to be a major risk factor for developing depression at some point of an individual's life (Monroe et al., 2014). Family studies have shown that the risk of MDD manifestation in the offspring of parents with MDD is more than 3-fold. A three-generation study identified that almost 60% of the grandchildren who had a family history of depression in two previous generations already had some psychiatric disorders at the mean age of 12 years.

Additionally, increased familial prevalence of MDD has been associated with an early age at onset and recurrence of the disease (Weissman et al., 2005).

Several genome-wide association studies (GWAS) have not yet yielded replicable findings for MDD (Bosker et al., 2011). This is explained by the small number of MDD cases evaluated for such studies, the clinical heterogeneity of the disorder, a modest heritability, the complexity of the genetic architecture for depression and interactions of genetic vulnerability with environmental susceptibility factors. However, a recent GWAS pathway analysis of over 60000 cases found significant associations between immune, neuronal signalling, synaptic density, histone cascades and psychiatric disorders, including MDD, suggesting a clustering of risk variants in these disorders (The Network and Pathway Analysis Subgroup of the Psychiatric Genomics Consortium, 2015).

The underlying genetic factors most explored have been the gene variants in the *SLC6A4* and *BDNF* genes. Results from studies that investigated the Val66Met polymorphism in the *BDNF* gene, coding for the protein with the same name being involved in a wide repertoire of neuronal functions, are mostly mixed (Lohoff, 2010). Findings in the meta-analysis that included 22 studies with a pooled total of 14233 participants suggest that the Met allele of BDNF Val66Met significantly moderates the relationship between stressful life events and depression (Hosang et al., 2014). A polymorphism in the serotonin-transporter-linked polymorphic region (5-HTTLPR) of the gene that codes for the serotonin transporter (SERT) has been found to affect the transcription rate of the gene, with the short (s) allele transcriptionally less efficient in removing serotonin from the synapse and returning it to the presynaptic neuron than the alternate long (l) allele (Karg et al., 2011). The pioneer epidemiological study of Caspi and colleagues in 2003 (Caspi et al., 2003) that was

numerously replicated afterwards (Eley et al., 2004; Zalsman et al., 2006) provided evidence that the less functional s allele is associated with increased stress sensitivity and therefore with higher vulnerability to depression. Interestingly, another study demonstrated that the responses of males and females carrying the short 5-HTTLPR allele to environmental stress factors go in opposite direction; females tended to develop depressive symptoms, whereas males did not (Sjöberg et al., 2006).

This brings up another predisposing factor for depression, which is being female, with a female:male prevalence ratio of MDD of around 2:1 (Angst and Merikangas, 1997; Kessler et al., 2003). Clinical data indicate that gonadal hormones have a significant impact on mood in women (Altemus et al., 2014). Interestingly, women are more likely to suffer from depression during episodes of marked hormonal fluctuations including the premenstrual, postpartum (Buttner et al., 2013) and perimenopausal periods (Tangen and Mykletun, 2008), that are concomitant with a decline in 17- β estradiol. These observations together with numerous data from animal models, where rats exhibit depressive-like behaviours upon withdrawal of 17- β estradiol (Stoffel and Craft, 2004) which is reversed by 17- β estradiol treatment (Galea et al., 2001), suggest that this hormone is necessary for mood regulation. However, some studies failed to reproduce these findings (Galea et al., 2002), likely due to differences in the dosage and duration of treatment. The biological effects of 17- β estradiol on emotional regulation are not surprising, since two intracellular oestrogen receptors (ER α and ER β) are prevalent throughout brain areas implicated in maintenance of mood, including the hippocampus. The activation of intracellular oestrogen receptors results in changes in gene transcription, whereas binding to membrane oestrogen receptors triggers activation of different signalling cascades (Solomon and Herman, 2009). While animal data indicate that the distribution of

central ER α and ER β is similar between females and males, mRNA and protein expression levels in some brain regions are higher in females (Orikasa and Sakuma, 2004). Presumably, the higher incidence of depression in women could be associated with frequent changes in female sex hormones profile.

Additionally, personality traits such as neuroticism, which is the tendency to experience diverse and relatively more intense negative emotions, have also been implicated as a risk factor for depression already a long time ago (Nolan and O'Malley, 1988) and reconfirmed later (De Moor et al., 2015).

1.1.3.2 Environmental risk factors for depression

Early life stress (ELS) is a broad term that encompasses such experiences in childhood as natural disasters, war, maltreatment, sexual or physical abuse, severe neglect as well as adverse family environment, such as maternal depression, parental loss or divorce (Nugent et al., 2011). ELS is an established predictor of a wide range of adverse life experiences across the lifespan, including depression. Preclinical models of ELS in laboratory animals have become increasingly popular in studies on the pathogenesis of affective disorders and the development of novel pharmacological approaches to these conditions (Molet et al., 2014). Maternal depression in pregnancy is another important risk factor for offspring depression in early adulthood. A prospective study identified that adult offspring exposed to maternal depression in pregnancy was 3.4 times more likely to have a depressive disorder (Plant et al., 2015). The evidence for the effects of maternal stress, depression and substance use on adverse neurodevelopmental outcomes for the child is substantial, and known as “foetal programming” (Dunkel Schetter and Tanner, 2012; Glover, 2014; Moisiadis

and Matthews, 2014). However, the number of longitudinal studies that have investigated the effects of various antenatal factors on the development of depression in adolescent or adult offspring is limited. Major findings on prenatal environmental exposures and foetal origins of depression from human studies are summarized in **Table 1**. Psychological stress at any time in life has been repeatedly linked to the onset of depression, being recognised now as a major risk and trigger factor for this condition (Cohen et al., 2007).

It is well established that all of the above aspects are important risk factors for depression, however one does not invariably lead to psychopathology. Partially, this is explained by gene-environment interactions where genetic differences influence the likelihood that exposure to different factors will eventually result in a mood disorder.

Study	Subjects	Main findings	Reference
Retrospective cohort study investigated psychiatric disorders exhibited by children with prenatal alcohol exposure	23 mother-child (5-13 years old) dyads	26% were diagnosed with major depressive disorder or adjustment disorder with depressed mood and 35% met criteria for bipolar disorder	(O'Connor et al., 2002)
Prospective-longitudinal study examined the association between prenatal alcohol exposure and child depressive symptoms, and the mediating effects of maternal and child characteristics	42 mother-child (4–5 years old) dyads	Structural equation modelling indicated that prenatal alcohol exposure was associated with higher levels of depressive symptomatology in children	(O'Connor and Paley, 2006)
Prospective-longitudinal study investigated whether antenatal maternal anxiety is associated with disturbances in HPA-axis regulation and whether the HPA-axis dysregulation mediates the association between antenatal maternal anxiety and depressive symptoms in post-pubertal adolescents	58 mother-child (14–15 years old adolescents) dyads	Regression analyses showed that antenatal exposure to maternal anxiety was in both sexes associated with a high, flattened cortisol day-time profile which, in female adolescents only, was associated with depressive symptoms	(Van den Bergh et al., 2008)
Cross-sectional cohort study investigated the association of prenatal psychosocial stress exposure with subsequent HPA axis regulation in adult life	31 adults	Healthy young adults whose mothers experienced severe stress during their pregnancy had significantly higher ACTH (adrenocorticotrophic hormone) in response to the Trier Social Stress Test and lower cortisol in response to a 1µg ACTH stimulation test in comparison with control group. Higher ACTH and	(Entringer et al., 2009)

		lower cortisol in response to ACTH stimulation are indicative of HPA axis dysregulation, constantly reported in depressed patients	
Prospective longitudinal community-based study researched whether antenatal depression predicts depression in adolescent offspring	125 mother-child (16 years) dyads	The risk of depression for the 16-year-olds exposed to antenatal depression was 4.7 times greater than for offspring not so exposed.	(Pawlby et al., 2009)
Prospective longitudinal investigation of associations between symptoms of antenatal and postnatal parental depression with offspring depression at age 18 years in a UK community-based birth cohort	8937 pregnant mothers and 4566 adolescents at age 18 years	Offspring were 1.28 times more likely to have depression at age 18 years for each standard deviation increase in maternal depression score antenatally, independent of later maternal depression.	(Pearson et al., 2013)
Prospective longitudinal study investigated whether there is an association between offspring exposure to maternal depression in pregnancy and depression in early adulthood	103 mother-child (18–25 years) dyads	Adult offspring exposed to maternal depression in pregnancy were 3.4 times more likely to have a depressive disorder.	(Plant et al., 2015)

Table 1. The evidence for foetal programming of depression.

1.1.4 Neuroprogressive nature of depression: introduction of the concept

Ultimately, the combination of environmental and genetic risk factors can lead to a depressive episode, and along with clinical symptoms, structural and molecular changes have been observed across many different studies. MDD is a multifactorial mood disorder with etiologically heterogeneous symptoms that has been proposed to have a neuroprogressive nature. Neuroprogression is considered a potentially progressive stage-related process of neurodegeneration that includes apoptosis, reduced neurogenesis, reduced neuronal plasticity and increased autoimmune responses (Moylan et al., 2013; Vaváková et al., 2015). The phenomenon of neuroprogression can be recognized on clinical, structural and biochemical levels in MDD. On a clinical level it is common for patients to have increasing number and length of depressive episodes, a progressive reduction in inter-episode duration, poor response to treatment with the time course of the disease and functional and cognitive decline (Moylan et al., 2013). This could be partially explained by the sensitization theory, suggesting that repeated administration of psychological or organic stressors causes progressive time-dependent amplification of neuronal responses leading to increased susceptibility to further stimuli (Ursin, 2014).

Different studies suggest that a history of depression is associated with an increased risk of developing cognitive dysfunction and eventually dementia (Caraci et al., 2010). A study that aimed to investigate whether the risk of developing dementia increases with the number of affective episodes in patients with MDD found that the rate of dementia increased by 13% with every depressive episode leading to hospital admission (Kessing and Andersen, 2004). A meta-analysis published a few years later, that included 20 studies aggregating data of 102172 individuals from 8 countries, showed that persons with a history of depression were more likely to be

diagnosed with Alzheimer's disease later in life. In addition to being a remote risk factor for Alzheimer's disease, depression may also be a prodromal feature of it (Ownby et al., 2006). The longest longitudinal study that covered 17 years of follow-up reported a 70% increased risk of developing dementia in individuals with depression (Saczynski et al., 2010), whereas as mentioned above, the last published meta-analysis found a twofold increased risk of developing dementia in patients with a history of late-life depression (Cherbuin et al., 2015).

It is now well recognized that decreased cognitive performance is a major contributor to the disability associated with the depressive disorder and it needs to be addressed with psychopharmacological therapy (Talarowska et al., 2014). A cognitive dysfunction in affective disorders suggests that brain structures responsible for emotional expressions and cognitive functions are linked to each other. One of the brain areas commonly affected in Alzheimer's disease patients and those experiencing mild cognitive impairment is the hippocampus (Mueller et al., 2010). Notably, neurodegenerative characteristics in this region of the brain is a consistent finding in depressed patients, who show decreased hippocampal volume (Videbech and Ravnkilde, 2004) along with structural alterations in the amygdala, orbitofrontal cortex, anterior cingulate cortex and basal ganglia. The role of the hippocampus which is implicated in the pathogenesis of depression and is relevant to this thesis will be described in further detail in section **1.1.6**.

There are a variety of neurobiological mechanisms that are common between depressive disorders and those of a neurodegenerative conditions such as dementia. Alterations at a molecular level, which together with diverse environmental influences contribute to the pathophysiology and neuroprogression of MDD, mediate changes in the brain chemistry and structure, including decreased adult neurogenesis, which will

be discussed in section **1.1.6.2**. In addition to impaired neurogenesis, major pathways include variations in neurotransmitter systems, hypothalamic-pituitary-adrenal (HPA) axis modulation, inflammatory alterations, epigenetic influences, mitochondrial dysfunction and OS (**Figure 1**). In particular, OS is one of the neurobiological mechanisms that hugely contributes to neurodegenerative processes (Gandhi and Abramov, 2012), and as described in section **1.3.1** it is commonly found to be increased in depressed patients. However, what happens at a molecular level in the brain of those depressive disorder patients is not clear (Balmus et al., 2016).

This thesis focuses on furthering the understanding of the role of OS in the pathogenesis of depression by modelling it in a relevant human *in vitro* system, investigating also if various compounds with antidepressant action are capable of preventing the damage inflicted by OS.

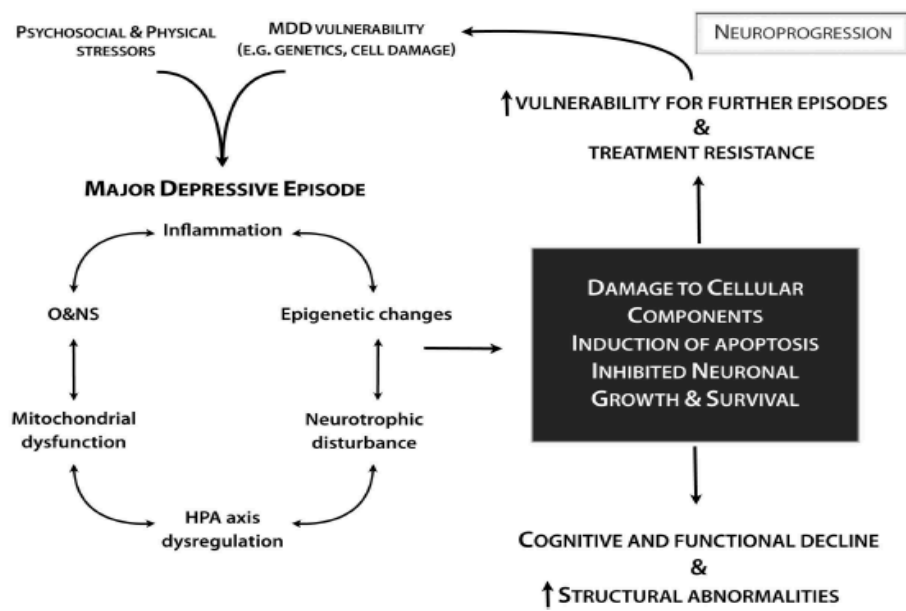


Figure 1. A combination of factors leading to a major depressive episode. A combination of psychological and physical stressors together with pre-existing vulnerability can cause a major depressive episode. Various dysregulated molecular mechanisms, including OS, can lead to the damage of cellular components, induction of apoptosis and reduced neurogenesis. These events can precipitate structural brain abnormalities, cognitive and functional decline, increased tolerance to treatment and vulnerability to future episodes (Moylan et al., 2013).

1.1.5 Monoamine deficiency theory of depression

Despite the evidence of several systems being involved in the pathogenesis of MDD as described above in 1.1.4, most currently available pharmacological treatments on the market target monoamine neurotransmitters. The monoamine theory of depression emerged serendipitously from clinical observations back in 1950s when clear mood-raising effects were noted in tuberculosis patients upon treatment with a tuberculostatic drug - iproniazid. Later it was shown that administration of iproniazid to experimental animals produced a rapid increase in brain levels of serotonin and had a significant stimulant effect on behaviour. The effects of iproniazid are mainly due to inhibition of the enzyme monoamine-oxidase-A (MAO-A) located at the level of the mitochondrial membrane, and capable of oxidative deamination of the biogenic amines, in particular adrenaline, noradrenaline and serotonin (López-Muñoz and Alamo, 2009). These monoamines are major neurotransmitters in the brain, responsible for signal transduction between the neurons. They are released from synaptic vesicles into the synaptic cleft where they bind to the receptors on the dendrites of other neurons (**Figure 2**). Along with iproniazid another drug, imipramine, that was initially used in psychotic patients but demonstrated antidepressant effects, was found to reduce the uptake of noradrenaline in the synaptic nerve endings. These psychopharmacological findings gave rise to the first biological hypotheses on the genesis of mental illnesses, known as the monoaminergic theory of depression. It was hypothesized that a primary cause of low mood is a functional deficiency of noradrenergic or serotonergic (or both) neurotransmission in certain brain areas (Pare and Sandler, 1959). On the bases of this monoaminergic theory over the last 60 years, alternative classes and generations of antidepressants were designed and introduced to the market, with higher selectivity, better safety profile and greater

convenience. However, all of them continue to share an action mechanism that revolves around the enhancement of aminergic functioning: inhibitors of noradrenaline and serotonin reuptake, antagonists of serotonergic 5-HT₂ receptors, specific noradrenergic and serotonergic antidepressants and selective inhibitors of noradrenaline reuptake. According to the British Association of Psychopharmacology guidelines the first line pharmacological treatment choices are selective serotonin reuptake inhibitors (SSRIs), due to better tolerability and safety in overdose (Cleare et al., 2015). Yet, there are still considerable issues such as the delay in the onset of antidepressant response or the percentage, estimated at around 30%, of patients who do not respond or partially respond to treatment to be addressed with regards to creating modern treatments. As research progressed in the neuropsychopharmacology field, a lot of non-monoamine-based actions have been explored for most of the antidepressants, such as immunomodulatory (Walker, 2013), neurotrophic (Berton and Nestler, 2006) and antioxidative effects (Behr et al., 2012). Based on these findings, the future of antidepressant therapy appears to be turning towards extraneuronal non-aminergic mechanisms or mechanisms that modulate the intraneuronal biochemical pathways.

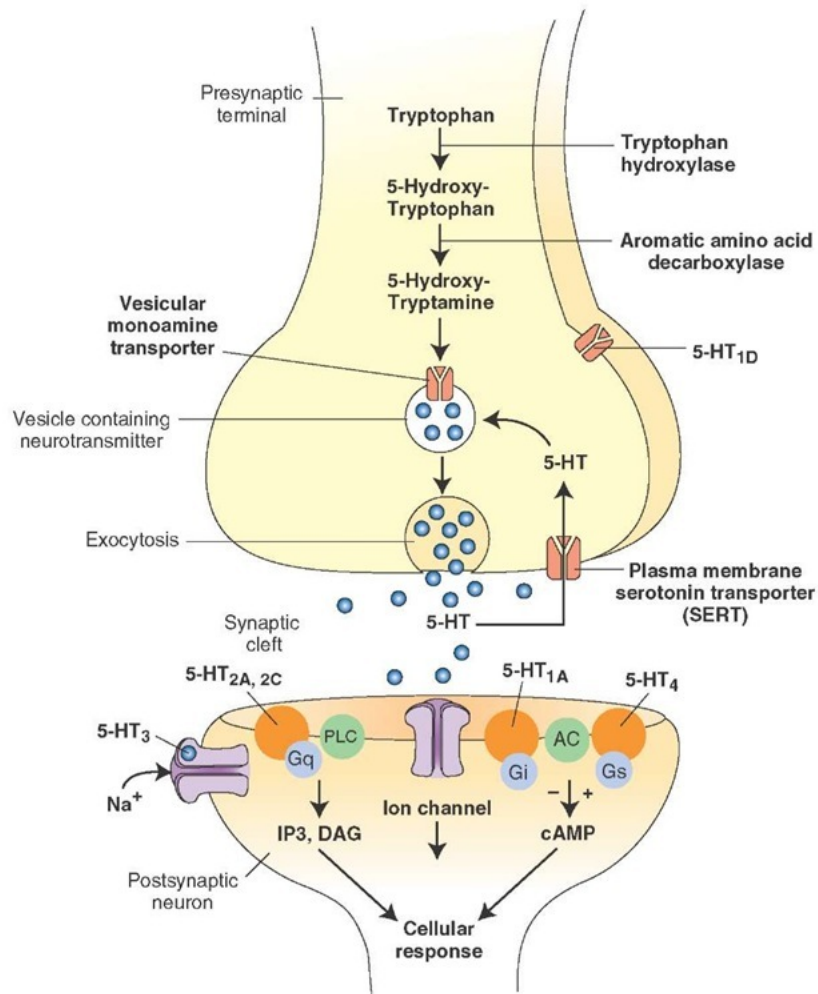


Figure 2. Serotonin neurotransmission. Serotonin or 5-hydroxytryptamine (5-HT) is synthesized from plasma tryptophan via hydroxylation and decarboxylation by the enzymes and stored in vesicles where it is ready for release. Once serotonin is released to the synaptic cleft it binds to various 5-HT receptors on the postsynaptic membrane and these leads to a cascade of events and cellular response. “Excessive” serotonin is removed by the reuptake mechanisms which involve active transport via plasma membrane serotonin transporter proteins (SERT) to the neuronal terminals where it is metabolised by MAO to 5-hydroxyindoleacetic acid. Adapted from Purves et al., 2004.

1.1.6 Hippocampus

The hippocampus is a paired structure of the brain located in the medial temporal lobe in humans and forms a part of the limbic system responsible for emotion processing, together with the amygdala and the olfactory cortex (Phelps, 2004). The hippocampus is a heterogeneous structure, with sub regions having different functions and developing along different growth trajectories. It is one of the most highly connected areas of the brain, and although it has traditionally been considered the “memory area”, it has started to emerge as a brain integrator of emotion and cognition. Behavioural, anatomical, and gene expression studies support a functional segmentation of the hippocampus into three compartments: dorsal, intermediate, and ventral. The dorsal or posterior hippocampus performs primarily cognitive functions whereas the ventral or anterior relates to stress, emotion, and affect (Fanselow and Dong, 2010). In healthy individuals, the anterior portion of the hippocampus, which interacts with the amygdala, prefrontal cortex and HPA axis and plays a role in anxiety-related behaviour, associative memory, and emotional processing, shows volume reduction with increasing age. Conversely, posterior regions (particularly, the CA3 region), which play a significant role in spatial learning and contextual memory, gradually increase over time (Gogtay et al., 2006). Anatomical connectivity and lesion studies point to discrete roles for these sub-regions. Additionally, the contribution of differential gene expression in ventral and dorsal hippocampus has been studied using microarray screening and *in situ* hybridization techniques aimed at identifying genes in the CA1 region of the adult murine hippocampus. Results in this study indicate the existence of CA1 sub-regionalized gene expression with known and unknown functions, where some genes exhibit a graded expression pattern across the dorsal–ventral axis, and others restricted to a discrete region (Leonardo et al., 2006).

Data from the Allen Brain Atlas project, which aims to understand the genetic structural and cellular architecture of the mouse brain by generating a genome-scale collection of cellular resolution gene expression profiles, shows that dorsal/ventral heterogeneity is very common with the top over-represented functional category within the regional gene set being cell adhesion. Furthermore, different neuropeptides were observed to be restricted to either dorsal or ventral hippocampus providing evidence for functional differentiation involving discrete signalling pathways (Lein et al., 2007).

Due to associations of the hippocampus with stress regulation and mood, this brain area has been implicated in affective disorders and has become the focus of depression research (Egeland et al., 2015).

1.1.6.1 Hippocampus in depression

Enormous amount of research implicates the hippocampus in the pathogenesis of MDD, clinically recognized as a highly stress-sensitive illness. Psychosocial stress, in turn, is associated with structural changes to the hippocampus, likely due to being densely populated with receptors for stress hormones that are released in response to stressful experiences and known to be neurotoxic (Snyder et al., 2011). In particular, it has been numerously demonstrated in animals that increased levels of corticosterone (analogue of cortisol, the major stress hormone in humans) induce depressive-like behaviour, hippocampal volume reduction, change in neuronal morphology and decreased neurogenesis in the hippocampus, that could be reversed by glucocorticoid receptor antagonists or antidepressants (Mayer et al., 2006; Murray et al., 2008; Sapolsky, 1985; Tata and Anderson, 2010). Additionally, human

conditions of chronic hypercortisolemia such as Cushing's disease, are associated with hippocampal atrophy and cognitive deficits that are reversible following normalization of cortisol levels (Starkman et al., 1999, 1992).

Reduction in hippocampal volume is consistently present in depressed patients with illness duration of more than two years and in those with more than one depressive episode (McKinnon et al., 2009). It has been reported in a meta-analysis that the total hippocampal volume is decreased in depressed patients for 8% on the left side and 10% on the right side, also showing a direct correlation between increasing episode number (Videbech and Ravnkilde, 2004) and length of untreated episodes (Sheline et al., 2003). It has been hypothesised that this hippocampal volume loss may explain the long lasting mood and memory disturbances which occur in depression (Gould et al., 2007; Sahay and Hen, 2007). In line with this notion, the domains of memory function that are found to be impaired in depressed patients are those that are most dependent on hippocampal function (MacQueen and Frodl, 2011).

The importance of the hippocampus in depression is also indicated by the finding that a larger hippocampal volume predicts a better response to antidepressant treatment (MacQueen et al., 2008), and successful antidepressant treatment can reverse the reduction in hippocampal volume in depression in humans (Sheline et al., 2003). It has also been noted that subjects at risk for MDD have reduced hippocampal volumes before the clinical onset of illness (MacQueen and Frodl, 2011) implying that molecular alterations might precede clinical signs. There is still no consensus regarding the nature of the underlying cellular changes that constitute hippocampal volume loss detected in depression (MacQueen and Frodl, 2011).

Connectivity studies have identified the hippocampus as one of several regions, including the dorsomedial and dorsolateral prefrontal cortex, and the anterior

cingulate cortex as components of a network that is dysregulated in MDD (Frodl et al., 2008). Additional evidence from neuroimaging, neuropathological and lesion analysis studies further implicates the hippocampus in the central nervous system networks normally regulating aspects of emotional behaviour (Drevets et al., 2008). Limbic–cortical–striatal–pallidal–thalamic circuits, including the prefrontal cortex, amygdala, hippocampal subiculum, ventromedial striatum, mediodorsal and midline thalamic nuclei and ventral pallidum are brain regions with which hippocampus has afferent and efferent connections (Miller et al., 2010).

In addition to the hippocampus being regularly found to be reduced in size in depression, implicated in the function of mood and emotional regulation, it is also the part of the brain most sensitive to environmental changes, particularly to stressful stimuli (Thomas et al., 2007). Both psychological and psychosocial stress have been associated with structural changes in the hippocampus (MacQueen and Frodl, 2011), reflecting the strong relationship between depression and environmental stressors (Kessler, 1997).

1.1.6.2 Adult hippocampal neurogenesis

Until recently the dogma was that the mammalian adult brain had no capacity to produce new neurons. However, the discovery of the formation of new born neurons out of neural progenitor cells, called “neurogenesis”, throughout adult life shifted the paradigm of brain development. There are two discrete neurogenic niches described in the adult mouse brain, the dentate gyrus of the hippocampus and the sub-ventricular zone (SVZ) of the lateral ventricles (Deng et al., 2010).

It takes approximately eight weeks for new born neurons to reach morphological maturity, with the number of spines reaching a plateau at this point with considerable connectivity to the local circuit (Song et al., 2012). During this period these new born neurons are highly plastic and regulated by neural activity and modulatory systems. The pioneering studies of Ernst and Frisen have affirmed the existence of adult neurogenesis in humans and demonstrated more extensive neuronal turnover than many had predicted (Ernst and Frisen, 2015). It has been estimated that 35% of the total numbers of hippocampal neurons are subject to turnover, with a median turnover rate of 1.75% per year, producing around 700 new born neurons each day, so that over the course of a lifetime hippocampal neurons subject to exchange come to make up the entire population of dentate gyrus (Spalding et al., 2013). In addition to neurogenesis in the hippocampus and SVZ, in humans newly generated cells were also found in the striatum, the brain area associated with motor control and various cognitive functions, such as reward cognition and motivation (Ernst et al., 2014). The process of adult hippocampal neurogenesis (AHN) has been extensively studied in animals and distinct developmental stages and markers have been identified (Kempermann et al., 2004). Interestingly, these studies have revealed that neurogenesis does not occur at a persistent low rate; instead, neurogenesis is a heavily regulated and dynamic process that is sensitive to environmental influence through positive inducers such as physical exercise, enriched environments, learning, certain dietary regimens (e.g. caloric restriction) and nutritional supplementation (e.g. polyphenols and poly-unsaturated fatty acids) and also by factors which have negative effects such as sleep deprivation and glucocorticoids associated with stress (**Figure 3**) (Aimone et al., 2014; Snyder et al., 2011). AHN represents a novel and powerful form of neuroplasticity and has been proposed to add a distinctive functionality to the

mammalian hippocampus whereby new neurons provide cognitive flexibility in the face of diverse environmental conditions (Clelland et al., 2009; Kempermann, 2012). AHN has been particularly implicated in depression, and considered to be essential for treatment and recovery from the illness (Lucassen et al., 2016), appearing to play a crucial role in the response to antidepressants. Experimental paradigms with animals demonstrate that exposure to acute or chronic stress reduces AHN and leads to the development of depressive-like symptoms (Schoenfeld and Gould, 2012), whereas enriched environment (Veena et al., 2009), exercise (Okamoto et al., 2015) or antidepressants (Duman et al., 2001) reverse these changes. Whether decreasing neurogenesis is enough in itself to induce depression is a subject of intense debate, mirroring the debate regarding the importance of neurogenesis in the action of antidepressants. It seems that the experimental reduction of neurogenesis in the absence of stress does not of itself induce depression, but rather that reduced neurogenesis contributes to the depression-like symptoms in animals by impairing stress modulation (Petrik et al., 2012).

The existing evidence on the involvement of the hippocampus in the pathogenesis of depression, together with the critical role of AHN on its development and recovery, make the use of the human hippocampal progenitor cell line capable of neurogenesis in this thesis of particular relevance to study molecular mechanisms underlining pathological process in MDD.

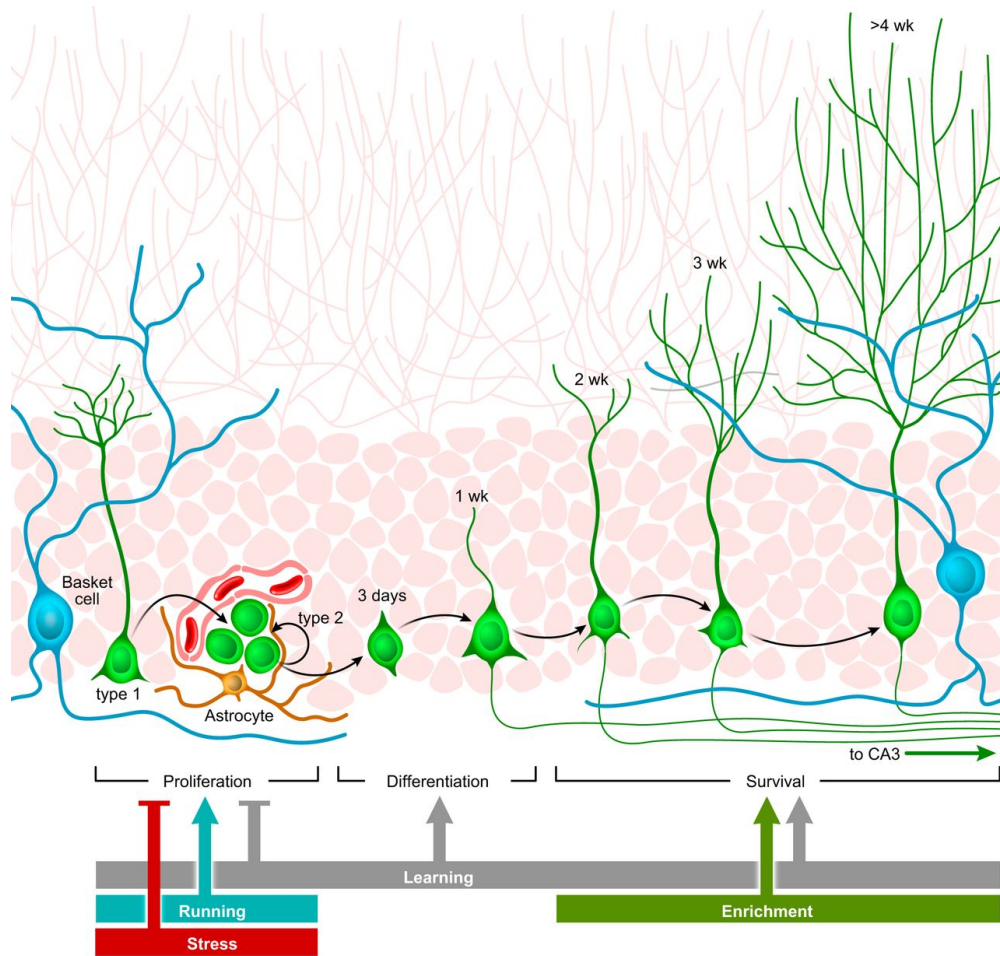


Figure 3. Development of the dentate gyrus granule cells to fully mature neurons. New neurons are derived from radial glial cells (type 1 cells) and are amplified more rapidly from neural progenitor cell (type 2 cells). During the next few weeks cells differentiate into neurons with dendrites and axonal projections (Aimone et al., 2014).

1.2 Oxidative stress and molecular mechanisms relevant to depression

1.2.1 Terminology in redox biology

In order to be consistent in using different terms of redox biology in this thesis, I would like to start with the definitions of the most common terms being reported in the literature.

Oxidants or oxidizing agents are chemical species that remove electrons from other species. This term can also refer to chemical species that transfer electronegative atoms, usually oxygen, to a substrate.

Reductants or reducing agents are elements or compounds that lose or "donate" electrons to other chemical species in a reduction-oxidation (*redox*) chemical reaction and hence become oxidized.

Free radicals are oxidants possessing unpaired electrons in their outer orbital and are capable of independent but very reactive existence. Most of the biological molecules are nonradicals, however when a free radical reacts with a nonradical, a new free radical is generated and this process can start a free radical chain reaction.

Reactive Oxygen Species (ROS) are a type of free radicals of particular importance for biological species derived from oxygen. ROS are highly reactive unstable ions that aim to react with other molecules to gain stability. Recently in the literature ROS has been used as a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals. The preferred practice should be to use the name of the identified species (Forman et al., 2015), however to maintain consistency, I will use the term ROS in the same manner, unless a particular species is known.

Antioxidants are species that protect biological targets against damage caused by ROS. They are usually substances that are present at low concentrations compared with those of an oxidizable substrate and which significantly delay or prevent the oxidation of that substrate (Halliwell and Gutteridge, 2015).

Total antioxidant capacity (TAC) (sometimes referred as total antioxidant activity/response) is an integrated parameter which means the cumulative capacity of free radical scavenging by the radical scavenging antioxidants contained in the test samples. This is often reported in clinical studies, since it is difficult to measure individual antioxidants (Niki, 2010).

Oxidative stress (OS) is an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage.

1.2.2 Oxidative stress and cellular responses

Under normal conditions the levels of oxidants are balanced by an antioxidative defence system, but when an imbalance between ROS and antioxidants occurs, OS leads to an alteration of biomolecules and the loss of control of the intracellular signalling pathways "redox related"; both will be discussed in detail below. OS can be mainly caused by two reasons: either due to excessive generation of ROS or insufficient production or activity of antioxidants. OS can result in injury to both cellular as well extra cellular components of the cell and can lead to cell death. ROS are mainly generated as a result of physiological intracellular metabolism in mitochondria and peroxisomes, involving a variety of cytosolic enzyme systems, in particular NADPH oxidases (NOX) that are widespread in the brain (Turrens, 2003). NOX2 is the most widely distributed isoform of this enzyme family in the central nervous system. There are seven known NOX isoforms that transport an electron from cytosolic NADPH to reduce oxygen to superoxide anion (**Table 2**) (Ma et al., 2017). The NOX and DUOX isoforms are structurally similar, with DUOX enzymes having an additional seventh transmembrane helix and possessing peroxidase activity (Bedard et al., 2007). The distribution and regulation of the NOX and DUOX isoforms throughout the body and brain varies depending upon the isoform. **Table 2** summarizes the reported distribution of NOX and DUOX isoforms in the central nervous system.

In addition, ROS are also produced in the brain as by-products of MAO activity, in a process which is vital to the inactivation of the monoaminergic neurotransmitters serotonin, dopamine, noradrenaline and adrenaline, involved in the pathophysiology of depression (Andreyev et al., 2005). ROS have different chemical properties and some of them are less reactive than others. For example, superoxide anion radical

($\cdot\text{O}_2^-$) reacts directly with few molecules in the human body and can pass through the anion exchange proteins in the plasma membranes, whereas hydroxyl radical ($\cdot\text{OH}$) can react with anything and when formed *in vivo* cannot migrate any significant distance within the cell (Halliwell, 2005). Notably, a response to oxidative injury may be reversible. Cells in an altered redox state do not necessarily lead to cell death, as after a variable period of time they can return to “normal”.

The consequences of OS largely depend on the tissue or cells being affected and on the levels of OS (**Figure 4**), therefore it is essential to study molecular mechanisms that involve OS in cell models relevant to the disorder. This research project addresses an issue of lack of studies of MDD pathogenesis in clinically relevant human models. Interestingly, some cell types, especially stem cells, respond to OS by increased proliferation, although moderate levels of OS often halt cell proliferation transiently. Adult and embryonic stem cells are exceptionally rich in antioxidants but their levels decrease as cells differentiate, presumably because some ROS are needed for stem cell differentiation. Generally, the cell's redox state with a more reducing environment is associated with cell proliferation, while a more oxidizing environment is associated with differentiation (Lewerenz et al., 2013). Mild and moderate levels of OS often cause increased upregulation of antioxidant defences and therefore cells adapt to these conditions. An elevated activity of antioxidants may increase resilience of the cells and completely or to some extent protect them from damage induced upon higher levels of OS imposed subsequently. Greater levels of ROS lead to oxidative damage of cellular components (lipids, DNA and proteins) and to mitochondria impairment, which promulgates the production of more ROS; this will be discussed in detail below (**1.2.3** section). OS can also cause cellular senescence when cells survive

but can no longer divide, due to increased levels of particular proteins that inhibit the cell cycle (Halliwell and Gutteridge, 2015).

Remarkably, adult neurogenesis, implicated in the pathogenesis of MDD, transiently generates OS, as was demonstrated both *in vivo* and *in vitro*. In young adult mice that were maintained under standard housing conditions and used for initial characterization of OS, the highest concentrations of oxidized DNA and lipid markers were found within the subgranular zone of the dentate gyrus, where production of new born neurons occurs. These observations were followed up *in vitro* to investigate if the constitutive cell division within the dentate gyrus was a source of OS. Undifferentiated neural stem cells (NSCs) from the SGZ were isolated and induced to terminally differentiate. During differentiation, intermediate precursor cells – which bridge undifferentiated neural stem and their postmitotic progeny – exhibited the highest levels of proliferation (as measured by thymidine analogue incorporation), pro-growth mitogen expression, cellular mitochondria and OS load compared to more- or less-differentiated counterparts (Walton et al., 2012). Using molecular imaging and a new selective fluorescent indicator for H₂O₂, it was demonstrated that adult hippocampal stem/progenitor cells generate H₂O₂ through NOX2 (an isoform of NADPH oxidase) to regulate intracellular growth signalling pathways, which in turn maintains their normal proliferation *in vitro* and *in vivo*. (Dickinson et al., 2011). In line with this, another study demonstrated that both exogenous and endogenous ROS can have a significant impact on neural stem and progenitor cell proliferation, self-renewal, and neurogenesis. Supplementation of H₂O₂ to culture media produced a modest increase in overall cell proliferation and a large increase in multipotent cells capable of producing neurons, astrocytes, and oligodendrocytes. When endogenous ROS levels were decreased in SVZ cells derived from NOX2 mutant mice, cells

displayed a significantly diminished NSCs self-renewal and multipotency, which was reversed by H_2O_2 addition. (Le Belle et al., 2011). Similar effects of OS on neurogenesis were observed in a study where rat primary neural progenitor cells (NPCs) exposed to H_2O_2 showed increase in neurogenesis and oligodendrogenesis (Pérez Estrada et al., 2014).

These data suggest that OS is produced as a result of routine adult neurogenesis and ROS are not only transit by-products of a highly energy intensive process, but also essential signalling molecules for normal cellular growth and further implementation of the cellular program. Therefore, in this thesis I sought to investigate how exogenously induced redox shifts might affect neurogenesis, exploring underlying molecular mechanisms.

Isoform	Product	Reported in CNS tissue
NOX1	Superoxide	<p>Cerebral cortex (Choi et al., 2015; Savchenko, 2013)</p> <p>Hippocampus (Choi et al., 2014; Skurlova et al., 2011)</p> <p>Cerebellum (Coyoy et al., 2008)</p> <p>Substantia nigra (Cristóvão et al., 2009)</p> <p>Striatum (Chéret et al., 2008)</p> <p>Hypothalamus (Guggilam et al., 2007)</p> <p>Cerebral vessels (Ago et al., 2005; Paravicini et al., 2004)</p>
NOX2	Superoxide	<p>Cerebral cortex (Bruce-Keller et al., 2010; Cooney et al., 2013; Serrano et al., 2003)</p> <p>Hippocampus (Dickinson et al., 2011; Hernandez et al., 2014; Le Belle et al., 2011; Serrano et al., 2003; Zhang et al., 2009)</p> <p>Cerebellum (Coyoy et al., 2008)</p> <p>Striatum (Guemez-Gamboa et al., 2011; McCann et al., 2008)</p> <p>Substantia nigra (Qin et al., 2013; Zawada et al., 2011)</p> <p>Hypothalamus (Guggilam et al., 2007; Ye et al., 2006)</p> <p>Subfornical organ (Lob et al., 2013)</p> <p>Lateral ventricle (Lelli et al., 2013)</p> <p>Subventricular zone (Lelli et al., 2013)</p> <p>Midbrain (Qin et al., 2013)</p> <p>Nucleus tractus solitaries (Wang et al., 2004)</p>
NOX3	Superoxide	Cerebral cortex (Cooney et al., 2013)
NOX4	Hydrogen peroxide (rapid	Cerebral cortex (Cooney et al., 2013; McCann et al., 2008; Vallet et al., 2005)

	conversion of NOX4-generated superoxide to hydrogen peroxide)	Hippocampus (Hernandes et al., 2014) Cerebellum (Coyoy et al., 2008) Hypothalamus (Guggilam et al., 2007; Infanger et al., 2010; Ye et al., 2006) Subfornical organ (Lob et al., 2013) Glioblastoma (Cheng et al., 2001)
NOX5	Superoxide	Glioblastoma (Antony et al., 2013; Cheng et al., 2001)
DUOX1	Hydrogen peroxide	None reported in CNS
DUOX2	Hydrogen peroxide	None reported in CNS

Table 2. Family of NOX enzymes and their distribution in the central nervous system. Adapted from Ma et al., 2017

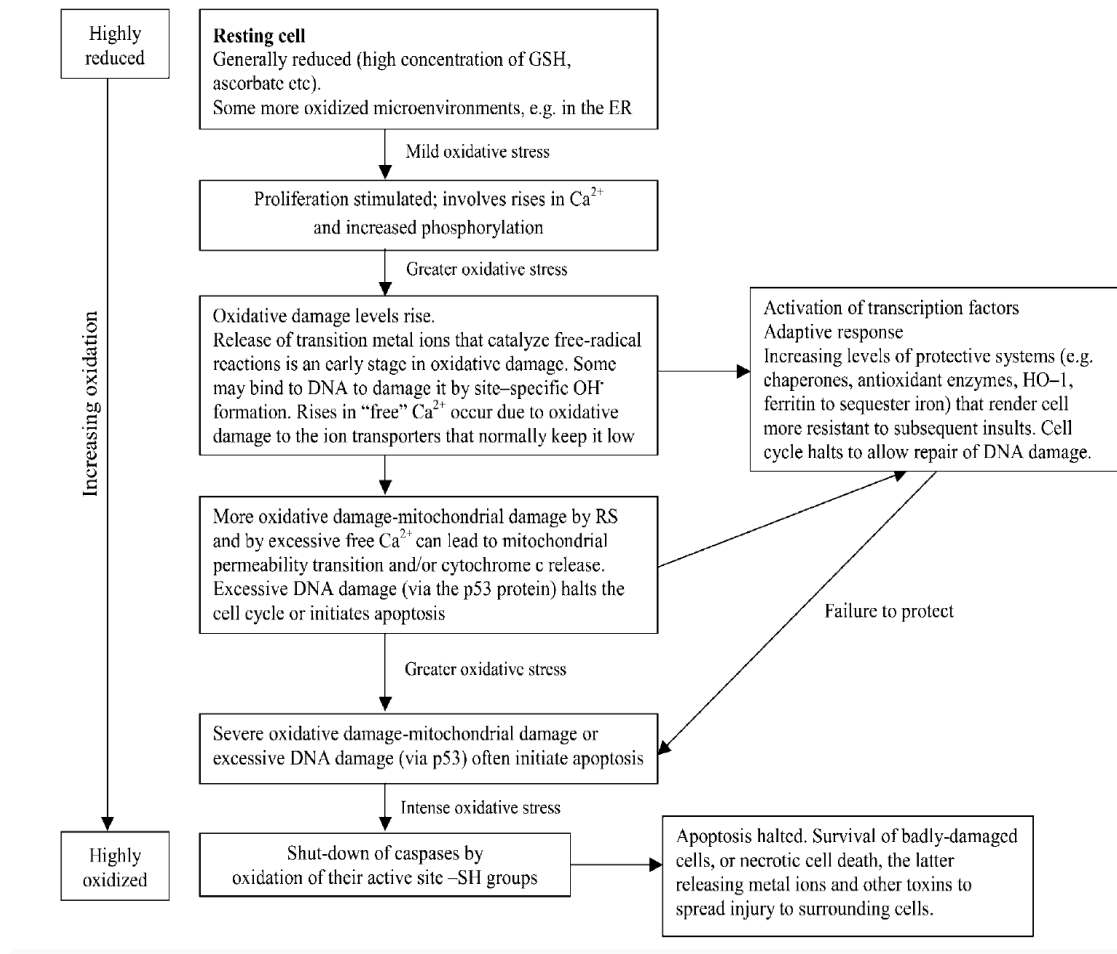


Figure 4. General cellular responses to different levels of OS (Halliwell, 2006).

1.2.3 Oxidative damage

The brain represents only ~2% of the total body weight and accounts for more than 20% of the total consumption of oxygen. Neurons and astrocytes, the two major types of brain cells, are largely responsible for the brain's massive consumption of O₂ and glucose (Gandhi and Abramov, 2012). Despite the essentiality of oxygen for neurons, some of its products such as oxygen radicals (e.g. superoxide, hydroxyl) and non-radicals (e.g. hydrogen peroxide) can be neurotoxic. Cells in the brain are especially vulnerable to the detrimental effects of OS, due to their high metabolic rate, the abundance of highly peroxidizable substrates and the modest antioxidant levels present. Excessive levels of ROS disrupt the neural cytoarchitecture and affect the function of a variety of biological molecules including lipids, nucleic acids and proteins, with inevitable damage inflicted to neurons (Friedman, 2011). Living organisms have evolved antioxidant defences to counterbalance OS and maintain redox homeostasis. These defence systems comprise nonenzymatic scavengers and quenchers, as well as enzymatic antioxidants, and include protection at the levels of prevention, interception and repair (Sies, 2013). The major enzymatic antioxidants are superoxide dismutases (SODs), catalase, and the family of glutathione peroxidases. In addition to these major enzymes, other antioxidants, including heme oxygenase, and redox proteins, such as thioredoxins, peroxiredoxins and glutaredoxins, have also been found to play crucial roles in the human antioxidant defences. Nonenzymatic antioxidants include low-molecular-weight compounds such as uric acid, melatonin, lipoic acid, coenzyme Q10, glutathione (GSH), β -carotene, flavonoids, polyphenols and vitamins A, C, D, and E. Some of them are not synthesised in humans and must be received with diet (Birben et al., 2012). Different antioxidants react with different ROS at variable rates, act in various locations and protect different molecular targets

(Halliwell, 2006), so it is important to identify antioxidant compounds that exhibit their properties in human brain cells in order to determine their potential therapeutics for MDD that is accompanied by increased levels of OS.

1.2.3.1 Lipid peroxidation

The oxidation of lipids is of particular importance due to the large amounts of polyunsaturated fatty acids (PUFAs) in the cellular membranes and organelles of brain cells (Innis, 2008). PUFAs contain two or more double carbon-carbon bonds and are easily oxidised in comparison with monounsaturated and saturated lipids. The initiation of lipid peroxidation can be caused by addition of ROS to the lipid, or by hydrogen atom subtraction from a methylene group (-CH₂-) of the lipid by a ROS (Chen et al., 2008). This can occur at different points of the carbon chain of the molecule. Importantly, a single initiation event lead to the propagation stage of lipid peroxidation and has the potential to generate multiple lipid peroxides by a chain reaction (Halliwell and Gutteridge, 1990). There are two broad outcomes to lipid peroxidation: structural damage to membranes and generation of oxidation-specific epitopes (Catalá, 2012). Lipid peroxidation has several altering effects on the structure and function of the membrane and ultimately on the cell. It decreases the viscosity of the lipid bilayer and increases membrane leakiness and therefore the molecules that normally do not cross the membrane other than through specific channels can pass. Eventually, membrane proteins are damaged and as a result various receptors, enzymes and transport protein are inactivated, ultimately leading to loss of membrane integrity. As a result of the continued oxidation and fragmentation of fatty acids, a variety of aldehydes and hydrocarbons are produced. Oxidized lipids

can have similar effects to hydrogen peroxide on the cells, stimulating or inducing proliferation depending on their concentration, and inducing apoptosis or necrosis at higher levels (Sevanian and McLeod, 1997). In addition to PUFAs, other molecules that contain double bonds such as vitamin A (retinol) or carotenoids can be also oxidized and at high levels serve as pro-oxidants.

1.2.3.2 End products of lipid peroxidation

End products of lipid peroxidation, including isoprostanes (IsoPs), malondialdehyde (MDA) and 4-hydroxy-2-trans-nonenal (4-HNE), are widely used as biomarkers of OS, despite some considerations about their validity. Lipid peroxidation products are cytotoxic, can act as antigens and promote immune responses, as they are recognized by the immune system as alien epitopes (Leonard and Maes, 2012). In mitochondria, the products of lipid peroxidation inhibit oxidative phosphorylation, compromise the permeability of the inner membrane, dissipate the mitochondrial membrane potential and reduce the Ca^{2+} buffering capacity, thus contributing to overall toxicity (Orrenius et al., 2007). These compounds also act as signalling molecules; for example, 4-HNE and IsoPs can rise cellular antioxidant defences, such as GSH and heme oxygenase 1 (HO-1) and activate the primary antioxidative defence transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Zhang et al., 2014).

IsoPs are prostaglandin-like compounds usually generated *in vivo* from the free radical-initiated peroxidation of PUFAs with at least three double bonds, such as linolenic acid, arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Roberts and Morrow, 2002). F2-IsoPs, the most studied class of IsoPs, are potent vasoconstrictors (Milne et al., 2015).

MDA largely arises from peroxidation of PUFAs with at least two double bonds and can attack proteins and therefore modify its functions and react with DNA bases introducing mutagenic lesions (Halliwell and Gutteridge, 2015).

4-HNE is one of many unsaturated aldehydes formed during peroxidation of omega-6 PUFAs, and it is especially toxic to neurons, by increasing calcium levels, inactivating glutamate transporters and damaging neurofilament proteins. 4-HNE is highly reactive and easily binds to biomolecules containing thiol and amino groups (Uchida, 2003).

All these lipid peroxidation end products have been found to be increased in depressed patients (Yager et al., 2010), as will be described in further detail in section **1.3.1**.

1.2.3.3 DNA and protein damage

Although DNA is a stable structure, it also suffers from the interaction with ROS and end-products of lipid peroxidation, by modification of purine or pyrimidine bases, single- and double-DNA breaks, damage to the 2-deoxyribose sugar, DNA-protein cross-linkage, and indirectly thorough damage to the DNA repair system. Both nuclear and mitochondrial DNA may suffer from OS insults and the biological significance of the oxidative damage depends on the extent of the damage, location in the genome and how fast it can be repaired. Oxidative damage of both nuclear and mitochondrial DNA are suggested to contribute to the pathogenesis of neurodegenerative disorders and depression (Maes et al., 2009; Tobe, 2013), and alterations in mitochondrial function have been proposed to be a potential target for depression treatment (Bansal and Kuhad, 2016). Mitochondrial DNA (mtDNA) is

mainly damaged due to mitochondrial dysfunction that results from increased OS, which further propagates the production of more free radicals and exacerbation of the OS cycle. It has been reported that mtDNA undergoes several-fold higher OS damage than nuclear DNA due to its proximity to ROS during electron transport and the absence of histone protection. In addition, since there are no introns in mtDNA, ROS are more likely to damage a gene than in nuclear DNA, although mtDNA is coated with proteins *in vivo* and some lesions can be repaired rapidly. Direct damage to DNA can introduce mutagenic lesions, some of which can also be repaired promptly enough before cell division. Otherwise, if lesions are not removed before DNA replication, mutations increase, eventually leading to programme cell death. Cells may respond to unrepaired DNA damage by silencing expression of the affected genomic region which may be vital for cell survival rather than by undergoing apoptosis. All these changes can affect transcription factor binding and lead to alterations of genetic regulation, then causing errors in translation and impairment of protein synthesis (Halliwell and Gutteridge, 2015).

Proteins can undergo direct attack by ROS or indirect deterioration following OS including end products of lipid peroxidation, such as MDA or 4-HNE. The identification of oxidised proteins as markers of OS *in vivo* is challenging, as all amino acids residues as well as all peptide bonds can be oxidised. Oxidative damage might occur to specific amino-acid residues, causing changes in the tertiary structure, degradation or fragmentation of the protein. Protein damage can be reversible, such as disulphide bond generation or glutathionylation, or irreversible, such as the oxidation of amino acid side-chains. In addition, OS can prevent protein synthesis by targeting initiation factors. Interestingly, this response might help in decreasing the synthesis of

bulk proteins under OS conditions in order not to flood the cell with damaged proteins (Halliwell and Gutteridge, 2015).

1.2.4 Glutathione (GSH) and system x_c^- as major regulators of redox homeostasis in the brain

Reduced glutathione (GSH) is a cysteine-containing tripeptide (γ -L-Glutamyl-L-cysteinyl-glycine) mainly present inside all cell types at millimolar concentrations and recognised to play a central role in intracellular endogenous antioxidant defences (Rizzo et al., 2010; Snoke and Bloch, 1952). GSH is a multifunctional molecule with diverse and still emerging functions that affect critical cellular processes. It serves several vital processes such as detoxifying electrophiles, scavenging free radicals, maintaining the essential thiol status of proteins, providing a reservoir for cysteine and modulating critical cellular processes such as DNA synthesis, microtubular-related processes, and immune function. In addition, GSH has been shown to modulate the activity of neurotransmitter receptors and proteins by post-translational modification (protein S-glutathionylation) (Kaplowitz et al., 1985; Lu, 2009; Meister and Anderson, 1983). GSH participates in redox reactions by reversible oxidation of its active thiol forming glutathione disulphide (GSSG), or by breaking the disulfide bridges formed inside and between proteins by the action of oxidants. The major provider of cysteine for GSH synthesis is the OS-inducible cystine/glutamate exchange system, known as system x_c^- , consisting of the 4F2 heavy chain (4F2hc) and the light chain, xCT/SLC7A11 subunits (Sato et al., 1999). These subunits are linked by a disulfide bond, with the regulation of xCT/SLC7A11 being an important determinant for the whole system. Importantly, the brain is one among the few organs

where system x_c^- is constitutively expressed in diverse areas, including the nucleus accumbens, the striatum, and the hippocampus (Sato et al., 2002). It is highly inducible by various stimuli, including OS and inflammation in an Nrf2-dependent or -independent manner. System x_c^- transports one molecule of cystine, the oxidized form of cysteine, into cells in exchange for one molecule of glutamate into the extracellular space (Bannai, 1986, 1984; Bannai and Tateishi, 1986) (**Figure 5 and 6**). Therefore, system x_c^- is critical for the maintenance of physiological redox conditions inside/outside of the cell and for modulation of extracellular glutamate concentrations (Lewerenz et al., 2013; Shih et al., 2006). Glutamate in turn is the most important excitatory neurotransmitter in the central nervous system and its excessive extracellular accumulation has been implicated in the pathogenesis of several neurodegenerative diseases and depression (Mehta et al., 2013; Sanacora et al., 2012).

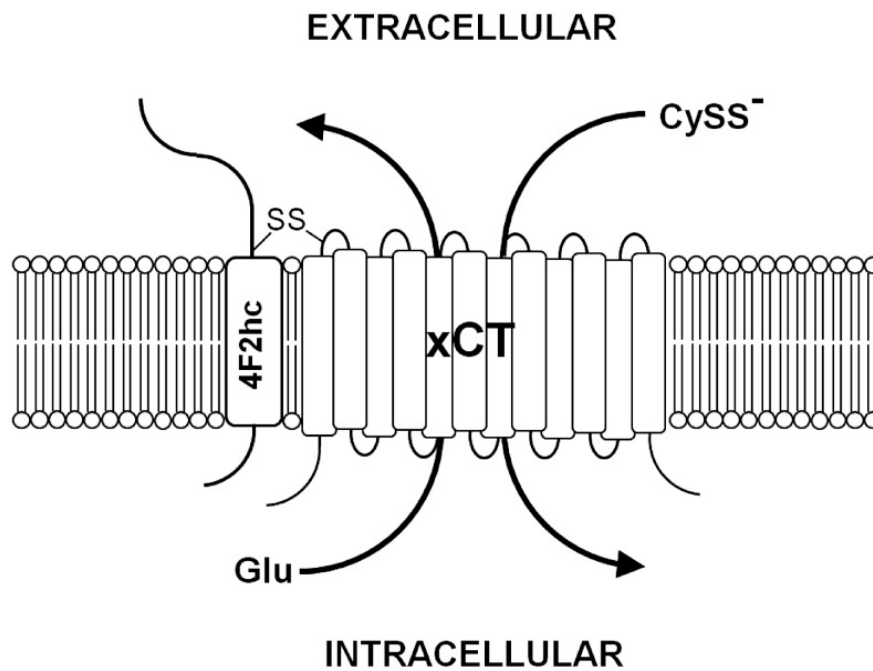


Figure 5. System x_c^- . System x_c^- is composed of the 4F2 heavy chain (4F2hc) and the light chain, xCT, which are linked by a disulfide bond (-S-S-). System x_c^- imports cystine ($CySS^-$) in exchange for glutamate (Glu) (Lewerenz et al., 2013).

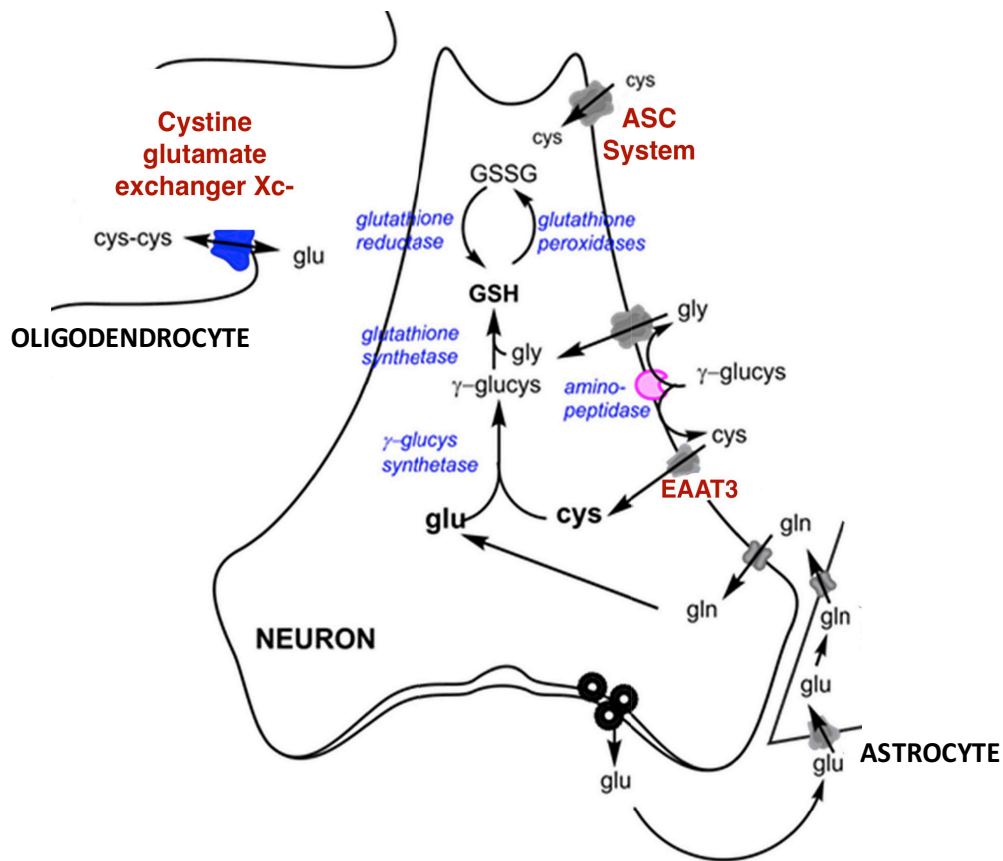


Figure 6. Mechanisms underlying transport of L-cystine via xCT and L-cysteine via other neutral amino acids transporters. Oligodendrocytes and astrocytes can take up cystine via the cystine/glutamate exchanger xc- system to supply cysteine for GSH synthesis. Into neurons most cysteine (80%) goes via the excitatory amino acid transporter 3 (EAAT3) with a small fraction supplied via alanine-serine-cysteine (ASC) system. GSH is converted to GSSH via redox reactions and is restored to GSH by GSSG reductase. Adapted from Rae and Williams, 2016.

1.2.5 Excitatory Amino Acid Transporter

Excess concentrations of glutamate are excitotoxic due to overactivation of ionotropic glutamate receptors and increase in calcium influx leading to neuronal death. Hence, glutamate concentration in the synaptic cleft is tightly regulated by the family of Excitatory Amino Acid Transporter (EAATs) 1-5, which are commonly observed to be down-regulated in many neuropsychiatric diseases (Lau and Tymianski, 2010; Sheldon and Robinson, 2007). Extracellular glutamate also acts as a competitive inhibitor for cystine uptake via system x_c^- completing the loop. It was demonstrated that transient overexpression of the neuronal EAATs increased intracellular GSH in the presence of high glutamate concentrations and protected hippocampal HT22 cells from oxidative glutamate toxicity. These effects were especially pronounced when EAAT3 was co-overexpressed with xCT leading to a concomitant decrease in the extracellular glutamate and increase in cystine uptake (Lewerenz et al., 2006). EAAT3 is a highly abundant, primarily neuronal transporter, with high densities at post-synaptic terminals protein that is widely expressed in CNS, with higher expression in the cerebral cortex, hippocampus, cerebellum and basal ganglia. Impairments in its function have been implicated in a variety of neurodegenerative diseases, including Alzheimer's disease (Bjørn-Yoshimoto and Underhill, 2016). EAAT3 plays an important role in glutamatergic termination in areas such as the hippocampus and in situations where glutamate reaches abnormally high extracellular concentrations, for example under ischemic conditions (Bianchi et al., 2014). This transporter, however, also contributes to controlling of the the cell redox potential due to its ability to transport cysteine (Chen and Swanson, 2003). It was demonstrated that EAAT3 knockout mice have a decreased neuronal glutathione level (Aoyama et al., 2006) and early onset brain aging, whereas *N*-acetyl-cysteine

(NAC) administration over four weeks significantly elevated glutathione levels in the cerebral cortex and hippocampus of mice and attenuated behavioural changes and decreased 4-HNE levels (Cao et al., 2012).

The oxidative glutamate toxicity resulting in cell death through activation of signalling pathways, rather than just an increase of ROS was described as a novel form of programmed cell death and named oxytosis (Tan, 2001). Interestingly, xCT knockout mice did not have a lower hippocampal glutathione content, increased OS or brain atrophy or cognitive impairments, however they had significantly lower extracellular hippocampal glutamate concentrations compared to wild-type littermates. Moreover, intrahippocampal perfusion with system x_c^- inhibitors lowered extracellular glutamate, whereas the system x_c^- activator NAC elevated extracellular glutamate in the rat hippocampus. These findings highlight that system x_c^- is not the only modulator of intracellular GSH and there are alternative mechanisms compensating for its deficiency to maintain the intracellular GSH pool *in vivo*. It also indicates that system x_c^- is important source of extracellular glutamate in the hippocampus (De Bundel et al., 2011).

Taken together, the cooperative action of system x_c^- and EAATs is necessary to ensure optimal protection of neuronal cells from OS by increased cystine uptake, GSH biosynthesis and properly controlled extracellular glutamate concentrations, processes that are known to be impaired in CNS illnesses with neurodegenerative patterns. Thus, I wished to explore if system x_c^- and EAAT3 might be involved in many potential cytoprotective effects of the compounds with antidepressant action under investigation in the thesis.

1.2.6 Cross talk between OS and inflammation in depression

No single event that happens in the living organism is isolated and changes in redox homeostasis are tightly linked to alterations in other various mechanisms linked to depression and in particular inflammation (Kim et al., 2016; Rawdin et al., 2013). Increased OS markers are often reported along with a neuroinflammatory signature in the blood of depressed patients, which is mainly represented by increased pro-inflammatory cytokines and acute phase proteins (Liu et al., 2016). Our mechanistic understanding of molecular pathways through which impaired redox homeostasis interacts with immune-inflammatory system in relation to MDD is not comprehensive. However, there are some distinct regulatory pathways linking inflammation and ROS production that are spread among transcription factors, proteins and small molecules, which I will describe below.

The generation of ROS is tightly regulated by inflammatory signals, such as the Negative Response ROS (NRROS) protein, highly expressed in immune organs and also detected in the brain. This protein is responsible for the degradation NOX2, one of the membrane-bound subunits of the NADPH oxidase complex, through which a great number of ROS are produced in response to inflammatory stimuli. The NRROS negative regulation of ROS production was shown in interferon- γ (IFN- γ) and lipopolysaccharide (LPS) -primed phagocytic cells and in NRROS-knockout mice (Noubade et al., 2014). Of relevance, downregulation of NRROS, previously known as Lrrc33 protein, led to increased levels of ROS, whereas knockdown of this enzyme in dendritic cells greatly increased nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation – a major inflammatory transcription factor in the absence of any inflammatory stimulation (Liu et al., 2013). Hence, one of the mechanisms by which inflammation might lead to increased OS and to further

propagation of the vicious cycle in depressed patients could be through down-regulation of NRROS by chronic inflammation, resulting in an increase in the levels of ROS and via a positive feedback loop, a further stimulation of the inflammatory cascade (**Figure 7**).

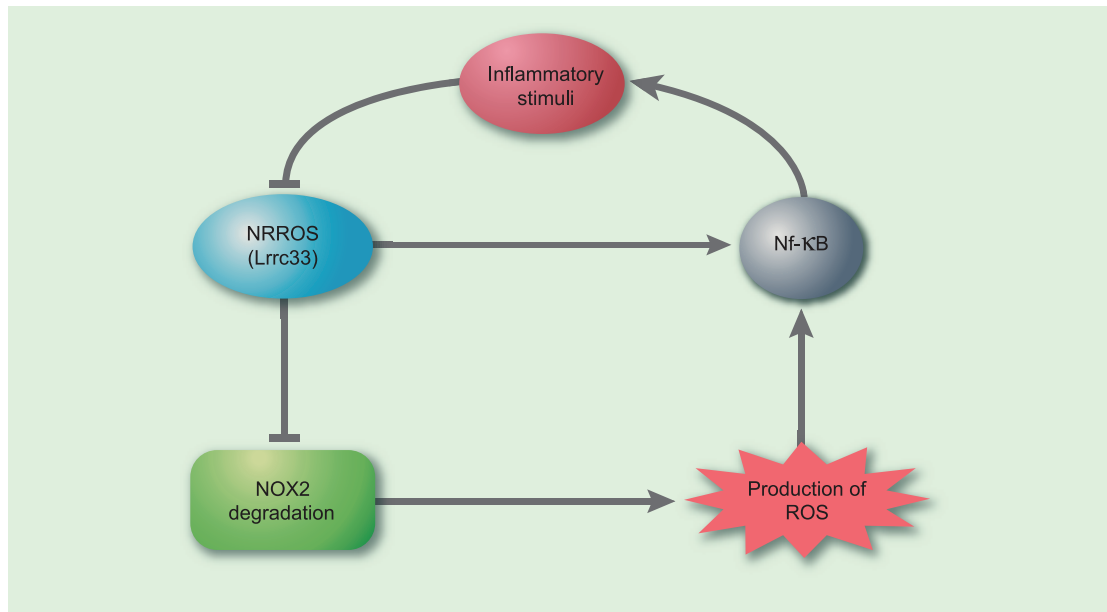


Figure 7. Proposed closed loop of inflammation and ROS production.

Inflammatory stimuli inhibit NRROS protein, in turn inhibiting the degradation of NOX2, which is responsible for ROS production. As a result, more ROS are produced in response to inflammation. ROS in turn activate NF-κB and further promote inflammation (Bakunina et al., 2015; Noubade et al., 2014).

ROS are necessary for the activation of the multiprotein inflammatory complexes known as inflammasomes, which are a major sensor of cellular stress signals. For example, the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome, the most extensively studied inflammasome, formed after the oligomerization of NLRP3 and subsequent recruitment of pro-caspase-1, increases the production of pro-inflammatory cytokine interleukin-1 β (IL-1 β) and activates NF- κ B, further promoting inflammatory cascades.

Importantly, as ROS change the chemical structures of different molecules, they generate a variety of modified oxidation-specific epitopes, which are highly immunogenic and can cause activation of adaptive immunity, such as immunoglobulin G (IgG) or IgM-mediated autoimmune responses. Indeed, significantly greater serum levels of IgG and IgM against oxidized low-density lipoproteins were reported in patients with major depression compared with controls (Maes et al., 2013). Furthermore, a separate study in serum of depressed patients detected significant reductions of IgA, which has a role as an immunomodulator helping to maintain the proper balance between pro- and anti-inflammatory states (Gold et al., 2012).

As many of the oxidation-specific epitopes have strong pro-inflammatory properties, including MDA, 4-HNE, thiobarbituric acid reactive species, 8-iso-prostaglandin-F2a and 8-hydroxydeoxyguanosine, they are considered as novel kind of damage-associated molecular patterns (DAMPs). DAMPs can initiate and perpetuate non-infectious immune responses as they bind to cellular and soluble pattern recognition receptors triggering inflammation. Conversely, some of the molecules that undergo oxidative modifications lose anti-inflammatory properties while many more are newly formed, with some possessing pro-inflammatory features. For example, PUFAs

inhibit prostaglandin E2 synthesis and modulate immune functions by regulating the production of a variety of cytokines, including IL-1, IL-6, tumour necrosis factor- α (TNF- α) and interferon- α (IFN- α), while upon oxidation these molecules acquire pro-oxidative and pro-inflammatory characteristics (Zunszain et al., 2012b).

The cascade of antioxidative and inflammatory events is orchestrated by several transcription factors and two key ones that are widely expressed in the central nervous system and researched in this thesis are nuclear factor Nrf2 and NF- κ B (Buelna-Chontal and Zazueta, 2013). The full dynamics of the interactions between Nrf2 and NF- κ B remain to be resolved and some important aspects of each are described below.

1.2.6.1 Nrf2 – major antioxidant transcription factor

Nrf2 is the major antioxidant transcription factor and the master regulator of the cellular defence pathway, which is activated in different cell types as a result of OS and other stimuli, leading to increased induction of the target antioxidants and enzymes that defend against oxidative damage (Mann and Forman, 2015). In addition, Nrf2 contributes to diverse cellular functions including differentiation, proliferation, inflammation and lipid synthesis. Interestingly, there is an increasing association of aberrant expression and function of Nrf2 in neurodegenerative diseases (Bryan et al., 2013). Under unstressed conditions Nrf2 is anchored in the cytoplasm through binding to Kelch like ECH associated protein 1 (Keap1), which degrades it by ubiquitination. Upon OS, Nrf2 travels to the nucleus where it forms a heterodimer with small Maf protein, binds to a DNA promoter antioxidant responsive element (ARE) and initiates transcription of genes that code for proteins that have

cytoprotective effects (**Figure 8**) (Itoh et al., 1999, 1997; Moi et al., 1994). Recent research has shown that oxidative and electrophilic stressors do not directly cause release of Nrf2 from Keap1, but rather conformational changes in the Keap1-Nrf2 complex that strengthen Keap1-Nrf2 binding leading to Nrf2 stabilization, its endogenous synthesis and increased binding to ARE sequence in the promoter of target genes (Baird and Dinkova-Kostova, 2011). In particular, Nrf2 inducers inactivate Keap1 via the modification of cysteine residues (Cys), (also known as “cysteine code” concept), Nrf2 is stabilized, and *de novo* synthesized Nrf2 translocates into the nucleus (Suzuki and Yamamoto, 2015). Thus, Keap1 turns out to be a sensor of electrophiles and ROS, and once a signal is detected it is transmitted for Nrf2 activation and induction of cytoprotective gene expression. Furthermore, degradation of Nrf2 is controlled by phosphorylation of one of its domains, which can be regulated by glycogen synthase kinase-3 (GSK-3), providing supplementary effects to the Keap1 pathway. It was shown that activation of GSK-3 in Keap1-null mouse embryonic fibroblasts (MEFs), or in human lung A549 cells that contain mutant *Keap1* by inhibition of the phosphoinositide 3-kinase (PI3K)–protein kinase B (PKB)/Akt pathway markedly reduced endogenous Nrf2 protein and decreased to 10–50% of normal the levels of mRNA for prototypic Nrf2-regulated enzymes (Chowdhry et al., 2013).

Nrf2 keeps under control the regulation of DNA damage recognition, repair and removal, as well as the modulation of proteasomes that are responsible for the degradation of damaged or misfiled proteins (Zhang et al., 2015). Major Nrf2 downstream proteins are NADPH dehydrogenase (quinone) 1 (NQO1), glutathione S-transferases (GST), heme oxygenase 1 (HO-1), antioxidant and related proteins, thioredoxins, γ -glutamate cysteine ligase (γ -GCS), glutathione peroxidase, glutathione

reductase, ubiquitination enzymes and proteasomes, and drug transporters (Niture et al., 2010). It also controls the levels of other key regulatory molecules, including protective proteins such as BDNF and the anti-inflammatory IL-10. Interestingly, it was shown that Nrf2 protects many different cell types by co-ordinately up-regulating classic ARE-driven genes, as well as cell type-specific target genes that are required for the defence system of each cell type in its unique environment (Lee et al., 2005). The broad functions of Nrf2 upon activation by OS have been shown with primary cortical neurons, where microarray data revealed that Nrf2 is important for the expression of immune and inflammation genes together with growth factors, signalling proteins and neuron-specific genes (Lee et al., 2003). Further support for the beneficial role of this transcription factor comes from mice studies where its deletion resulted in depressive-like behaviour, reduced levels of dopamine and serotonin and increased levels of glutamate in the prefrontal cortex (Martín-de-Saavedra et al., 2013). Interestingly, treatment of these Nrf2 knockout mice with the anti-inflammatory drug rofecoxib reversed their depressive-like behaviour, suggesting the involvement of Nrf2 in the induction of inflammatory cascades. The follow-up study based on the same Nrf2 knockout model of depression that used liquid chromatography–mass spectrometry to monitor neurotransmitter and metabolite levels found significantly decreased levels of another chief inhibitory neurotransmitter, γ -Aminobutyric acid (GABA), in various areas of the rat brain (Wojnicz et al., 2016). Conversely, inflammation around birth may have long-term detrimental effects on the Nrf2 system. As such, prenatal inflammation of rodents by exposure to LPS resulted in a dysfunctional Nrf2 response in adulthood, indicated by lower levels of GSH and a decrease in the activity of GSH synthase (Zhu et al., 2007). Due to the vast variety of these protective effects, Nrf2 has been suggested as a promising target to counteract

ROS mediated damage in neurodegenerative diseases and depression (Bakunina et al., 2015).

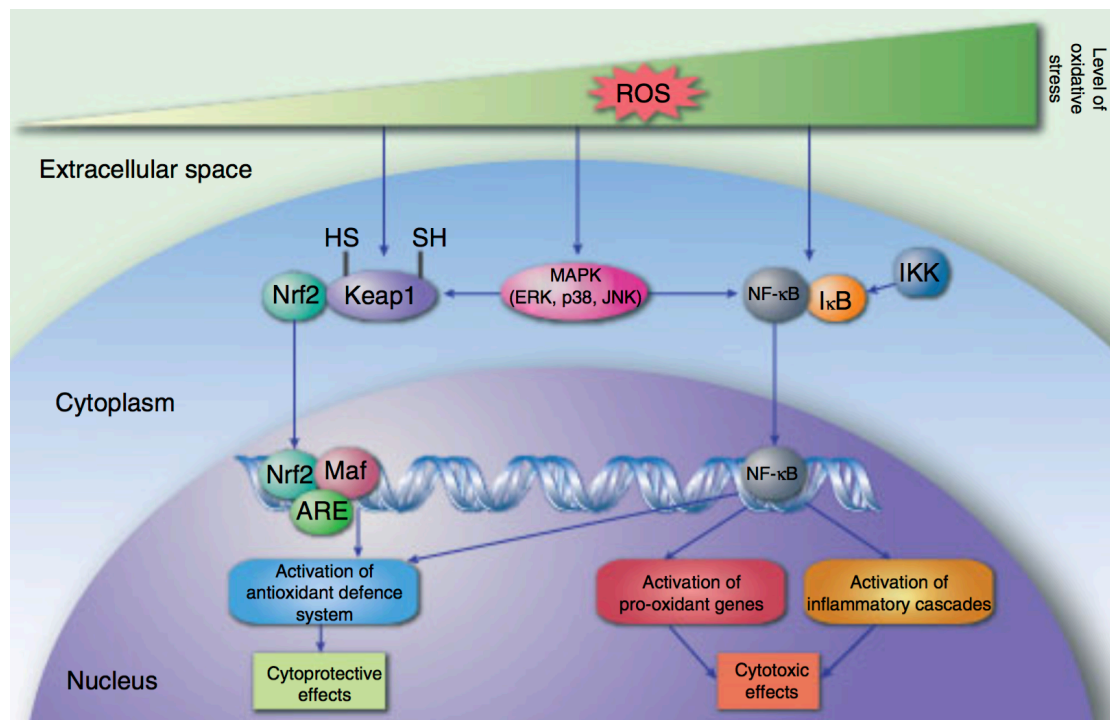


Figure 8. Activation of transcription factors by ROS. ROS can activate different transcription factors directly and/or through kinases. At low levels of OS, Nrf2 is activated: it is released from the cytoplasm and translocates into the nucleus where it forms a heterodimer with small Maf protein, binds to a DNA promoter Antioxidant Responsive Element (ARE) and initiates transcription of antioxidative genes, leading to cytoprotective effects. At higher levels of OS, NF-κB is activated: IκB is phosphorylated by IKK and as a result NF-κB is released from the cytoplasm and translocated into the nucleus. Depending on the cellular context, NF-κB can activate inflammatory cascades, pro-oxidant or antioxidant genes (Bakunina et al., 2015; Gloire et al., 2006; Itoh et al., 1999, 1997; Son et al., 2011).

1.2.6.2 NF- κ B – a redox sensitive transcription factor

NF- κ B is a transcription factor that has been widely implicated in the inflammatory hypothesis of the pathogenesis of MDD (Miller and Raison, 2016). The heterodimeric protein NF- κ B is identified as highly redox sensitive and involved in the control of a large number of physiological cellular processes, such as immune responses, cellular proliferation and migration, differentiation and survival and cell death programs. Under normal conditions it is inactive in cellular cytoplasm, where it is bound to the inhibitory protein I κ B. Upon ROS stimulation (or another stimulus, e.g., cytokines) I κ B is phosphorylated by I κ B-kinase and as a result p50/p65 NF- κ B dimers are released from the cytoplasm, translocate into the nucleus, bind to its DNA target sequence and initiate gene transcription. This NF- κ B pathway activation is recognised as the canonical or classical pathway. The noncanonical or alternative pathway differs from the canonical pathway in that only certain receptor signals can activate it and it leads to liberation of the p52/RelB NF- κ B complex (Gilmore, 2006) (**Figure 9**). Whereas oxidants enhance NF- κ B nuclear translocation and consequently its activation, the direct oxidation of NF- κ B decreases its DNA binding activity and therefore transcription of genes. Although NF- κ B is commonly associated with triggering pro-inflammatory cascades, it can also target antioxidant genes, as well as promote ROS production, which has physiological relevance. Furthermore, ROS can modulate NF- κ B activity both positively and negatively depending on the context. For example, activation of c-Jun N-terminal kinase (JNK) by TNF- α , contributing to inflammatory responses requires the generation of ROS, and this process can be counteracted by NF- κ B through the induction of genes that encode antioxidant enzymes such as manganese-superoxide dismutase (MnSOD) and ferritin heavy chain (Nakano et al., 2005; Perkins, 2007).

Of note here, the vast majority of studies concerning oxidant-induced NF- κ B activation have used H₂O₂ as a direct source of ROS. However, studies suggest that NF- κ B activation by H₂O₂ is highly cell-type specific and involves quite different mechanisms. This is even more true for NF- κ B activation by cytokines and OS, where the mechanisms and consequences of its activation are not comparable (Gloire et al., 2006).

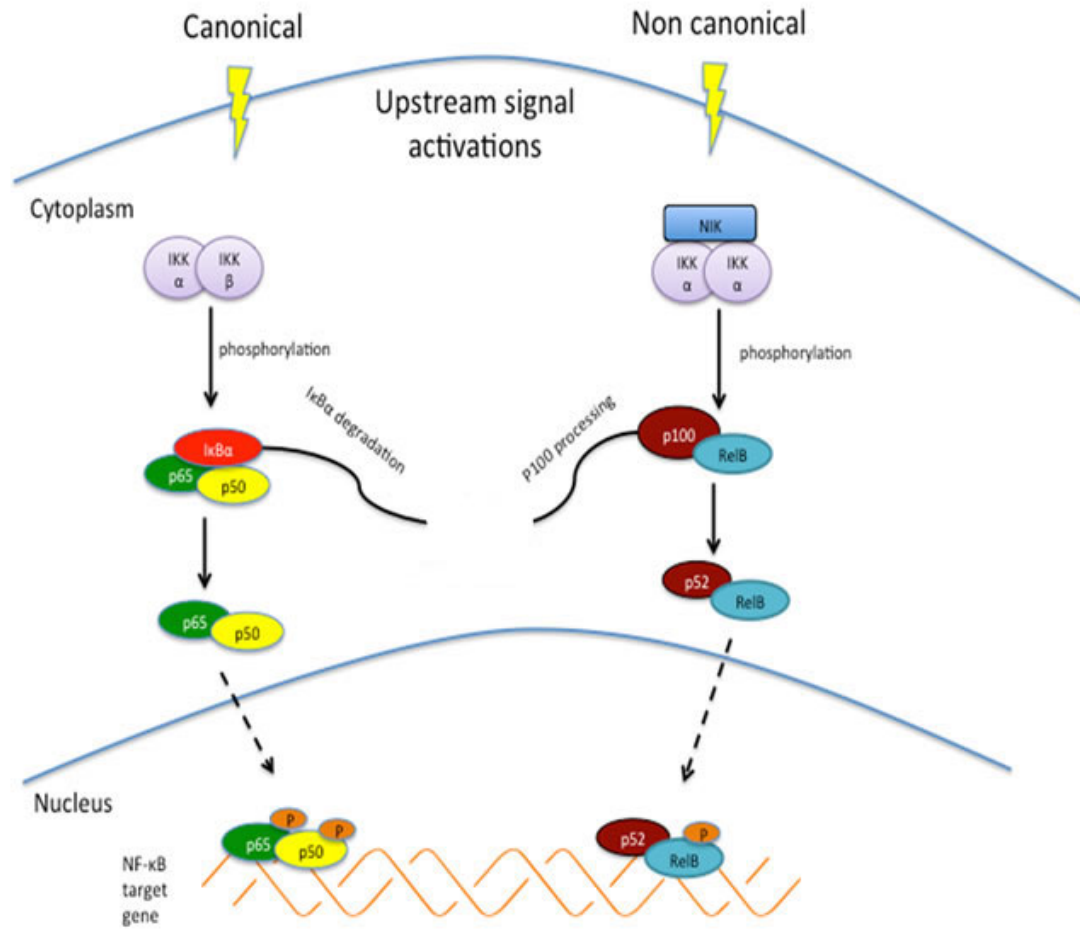


Figure 9. Canonical and non-canonical NF- κ B signalling pathways. Adapted from Zhou et al., 2015.

1.2.6.3 Kynurenine pathway affected by OS

Among other potential mechanisms that are involved in the pathogenesis of MDD and are linked to both increased OS and neuroinflammation is the kynurenine pathway (Maes et al., 2011). The pathway starts from the amino acid tryptophan (TRP), a serotonin precursor, which is metabolized to kynurenine (KYN). This transformation is catalysed by indoleamine 2,3-dioxygenase (IDO), an enzyme inducible by pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-2 and IL-1 β , and also directly by ROS (Ocampo et al., 2014). Notably, one of the major products of direct tryptophan oxidation identified *in vivo* is KYN. KYN may be further metabolized to either kynurenic acid (KYNA), a potentially neuroprotective metabolite, or to excitotoxic free radical generator compounds including 3-hydroxykynurenine (3-HK) and quinolinic acid (QUINA), whose increased levels, together with increased IDO, have been associated with MDD (Gabbay et al., 2010; Savitz et al., 2015) (**Figure 10**). Thus, OS contributes to the perturbation of the kynurenine pathway that has been implicated in the pathogenesis of depression and neurodegeneration (Lovelace et al., 2016). Additionally, the formation of new epitopes contributes to inflammation and to pathogenic inflammatory pathways postulated to underlie depression, such as these tryptophan catabolites produced by the kynurenine pathway.

The kynurenine pathway has two main relevant outcomes to these conditions: the reduction of available serotonin, putatively linked to increased metabolism of tryptophan into KYN by activated IDO, and the increased neurotoxicity due to the relative balance of QUINA and KYNA (Maes et al., 2009c; Myint et al., 2007; Savitz, 2016; Wichers et al., 2005).

These pathways also affect hippocampal neurogenesis. Indeed, tryptophan-2,3-dioxygenase (TDO) knockout mice show increased neurogenesis (Kanai et al., 2009). Recently, our lab demonstrated that treatment with the pro-inflammatory cytokine IL-1 β reduces neurogenesis in the human hippocampal progenitor cells that are also utilised in this thesis, by a mechanism involving a shift in the balance of the kynurenine pathway towards neurotoxic metabolites (Zunszain et al., 2012a). Therefore, I further investigated if redox shifts lead to alterations in the kynurenine pathway in my OS model.

OS and inflammation are intimately linked with each other in the pathophysiology of depression. Molecular consequences of OS that are hypothesized to take place in the pathophysiology of depression are summarized in **Figure 11**.

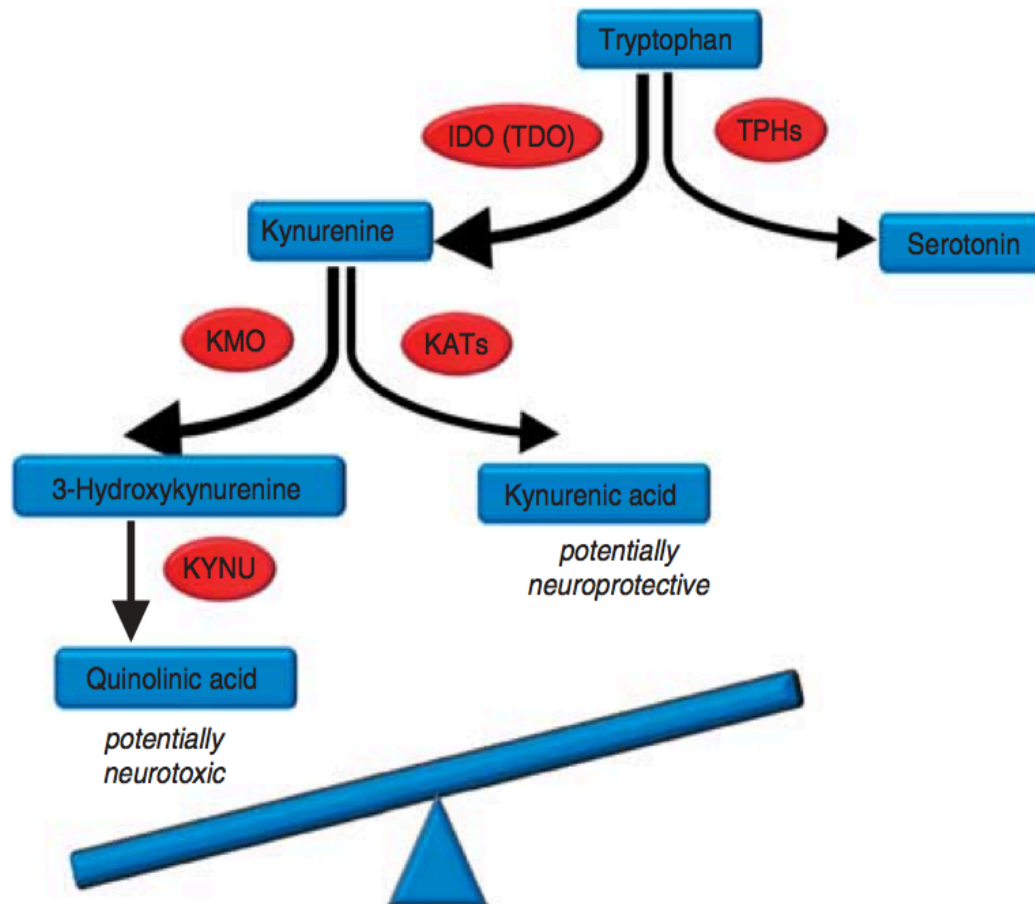


Figure 10. Simplified kynurenine pathway of tryptophan metabolism (Zunszain et al., 2012a).

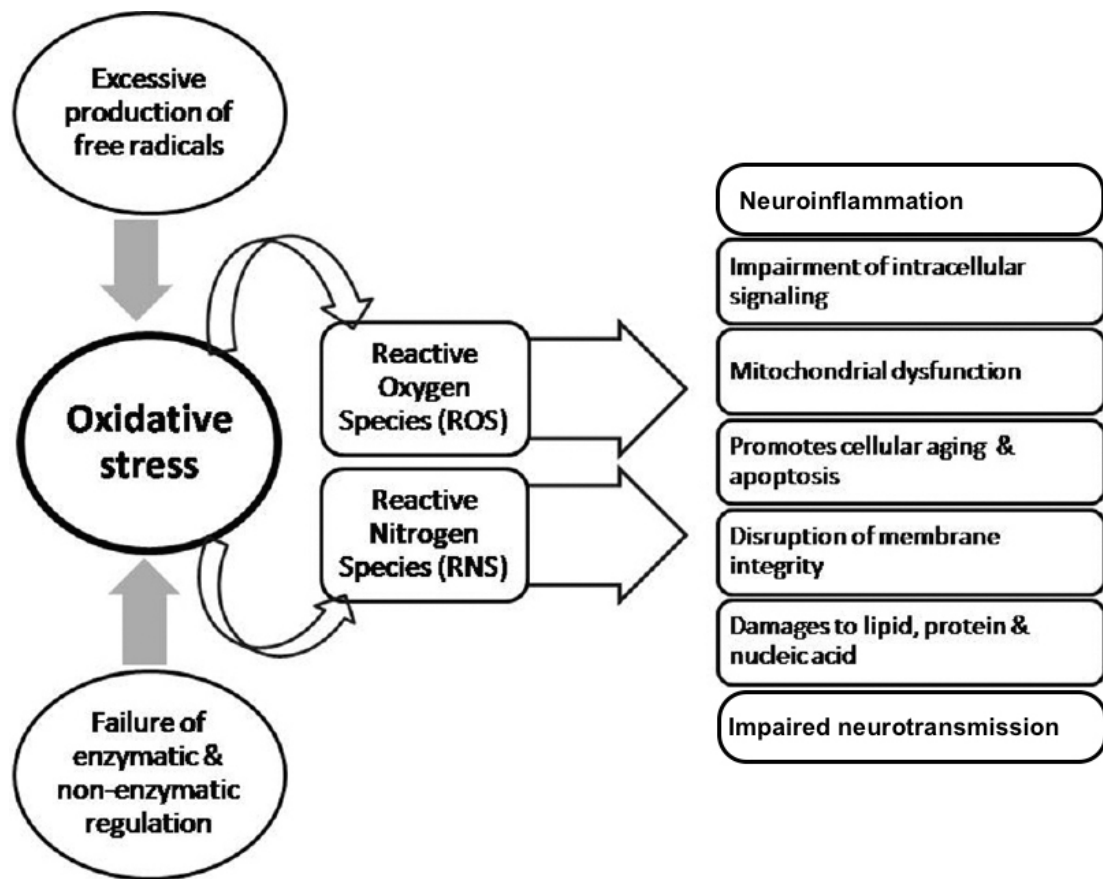


Figure 11. Schematic molecular consequences of OS that are hypothesized to take place in the pathophysiology of depression. Adapted from Kar and Choudhury, 2016.

1.2.7 Redox signalling

The generation of ROS within physiological levels is necessary to maintain redox homeostasis in a living organism. Interestingly, although ROS have been conventionally considered as toxic by-products of cellular metabolism, they also act as critical secondary messengers and essential elements of fundamental neurobiological processes such as cell growth, proliferation and differentiation, signalling, migration and adhesion, immune responses, biological synthesis, regulation of gene expression and regulated forms of cell death. Additionally, ROS are crucial molecules in fighting bacterial agents causing infections (Nathan and Cunningham-Bussel, 2013). Therefore, although the antioxidant defence network must minimize the levels of most ROS, it still needs to permit enough to remain for their essential roles (Halliwell and Gutteridge, 2015). The activation of various molecular pathways by OS provides numerous examples of cell and tissue specificity and demonstrates the complexity of the mechanism governing its activation. As introduced above, ROS cause post-translational protein modifications in a variety of ways, and the cysteine residues that are the most susceptible to oxidation. Thiol transformations are particularly important because they are reversible and therefore of great physiological relevance in cellular signalling.

ROS also affect the activities of serine/threonine kinases and the best known of these are members of the mitogen-activated protein kinase (MAPK) family (Maher and Schubert, 2000). The transformations of MAPKs, including extracellular signal-regulated kinase (ERK), JNK and p38, are most critical to cell fate and were researched in this thesis. These MAPKs are modulated upon diverse stimuli including OS, whereas antioxidants and inhibitors of ROS producing enzymatic systems, such as NOX2 block their activation. Each subgroup of MAPKs is activated through a

cascade of sequential phosphorylation events, beginning with the activation of MAPK kinase kinases (MKKK or MAP3Ks), followed by downstream activation of dual specificity MAPK kinases (MAP2Ks), which in turn stimulate MAPK activity through dual phosphorylation on threonine and tyrosine residues (**Figure 12**) (Son et al., 2011).

ERKs are often activated by growth factors and are primarily involved in cell proliferation and differentiation, whereas JNK and p38 MAPK are primarily activated in response to pro-inflammatory cytokines and environmental stress and are implicated in inflammatory responses, cell cycle arrest, DNA repair and cell death (Maher and Schubert, 2000). Interestingly, ERK1/2, p38 and JNK have been indicated as potential kinases that promote NF- κ B and Nrf2 activation (Buelna-Chontal and Zazueta, 2013). All three members of the MAPK family potentially can be activated by redox stimulus, however kinase activation may be cell type dependent. Therefore, it is important to study how MAPKs activity is modulated upon OS in human brain cells in order to reveal potential molecular redox-related mechanisms involved in the pathogenesis of depression.

It was demonstrated that direct exposure of rat cerebral cortex neurons to exogenous H₂O₂, used to mimic OS, has led to activation of MAPK pathways (Wang et al., 2003), while pre-treatment with NAC of rat pheochromocytoma and human neuroblastoma cells abrogated activation of MAPKs and neuronal death (Chen et al., 2009). The mechanisms by which ROS can activate MAPK pathways and the precise molecular targets of ROS in these cascades are not well defined. Plausible mechanisms include oxidative modifications of MAPK signalling proteins and inactivation of MAPK phosphatases that dephosphorylate and deactivate MAPKs (Son et al., 2011). Depending on the site of modification, products of these

transformations can inhibit or stimulate kinase activity in a similar way, as happens through phosphorylation. Whether these pathways are activated or inhibited and which specific ROS leads to modulation of its activity mostly depend on the cell type and the level of OS. Interestingly, not only ROS can activate MAPKs, but pathophysiological concentration of 4-HNE, a by-product of lipid peroxidation, could also increase the phosphorylation/activity of the major three MAPK pathways (Forman et al., 2008).

Of relevance to depression, post-mortem studies have reported decreased levels of ERK pathway molecules (Raf, MEK and ERK) in the hippocampus of depressed suicide subjects (Dwivedi et al., 2006; Yuan et al., 2010). An increased expression of a negative regulator of MAPK/ERK signalling, MAP kinase phosphatase 1 (MKP1) was observed in post-mortem hippocampus of depressed subjects and in chronically stressed rodents hippocampus, which was normalized by antidepressant treatment (Akbarian and Davis, 2010; Duric et al., 2010). Further studies have demonstrated that ERK signalling is reduced by chronic stress and reversed by fluoxetine (First et al., 2011), and that blockade of ERK signalling produces depressive and anxiety behaviours in animal models of depression (Qi et al., 2009). Conversely, acute administration of a MAPK inhibitor that inhibits ERK phosphorylation produced depressive-like behaviour and blocked the antidepressant-like effects of desipramine and sertraline in a mouse behavioural model for depression (Duman et al., 2007).

In contrast to these findings, some studies report that inhibition of ERK activation upon OS attenuated neuronal death and cellular injury, indicating a pro-death role for ERK1/2 (Sato et al., 2000). The activation of p38 and JNK MAPK that is mainly associated with a stress response has been demonstrated in animal models of depression (Meller et al., 2003) and depressed individuals (Felger et al., 2011). In

particular, administration of LPS to IL-1 receptor deficient mice lead to activation of p38 MAPK pathways, which enhanced brain serotonin transporter activity. This effect was abrogated by blocking p38 MAPK with an inhibitor (Zhu et al., 2010). Similarly, social defeat stress produced depressive-like behaviour in wild-type mice, but not in mice having p38 α MAPK selectively deleted in serotonin-producing neurons of the dorsal raphe nucleus. In addition, the stress-induced activation of p38 α MAPK translocated the serotonin transporter to the plasma membrane and increased the rate of transmitter uptake at serotonergic nerve terminals (Bruchas et al., 2011).

Thus, changes in redox homeostasis influence cell behaviour via complex mechanisms that involve transmission of the signal through activation of MAPKs, which may result in altered mood. Therefore, considering the possible role of OS in the pathophysiology underlining MDD, redox biology may provide further targets for pharmacological intervention. Therefore, relevant models to study molecular mechanisms are necessary, as there are a lot of exceptions to theoretical paradigms, partially due to the differences in the signal transduction machinery as well as variances in antioxidant control mechanisms existing in different cell types.

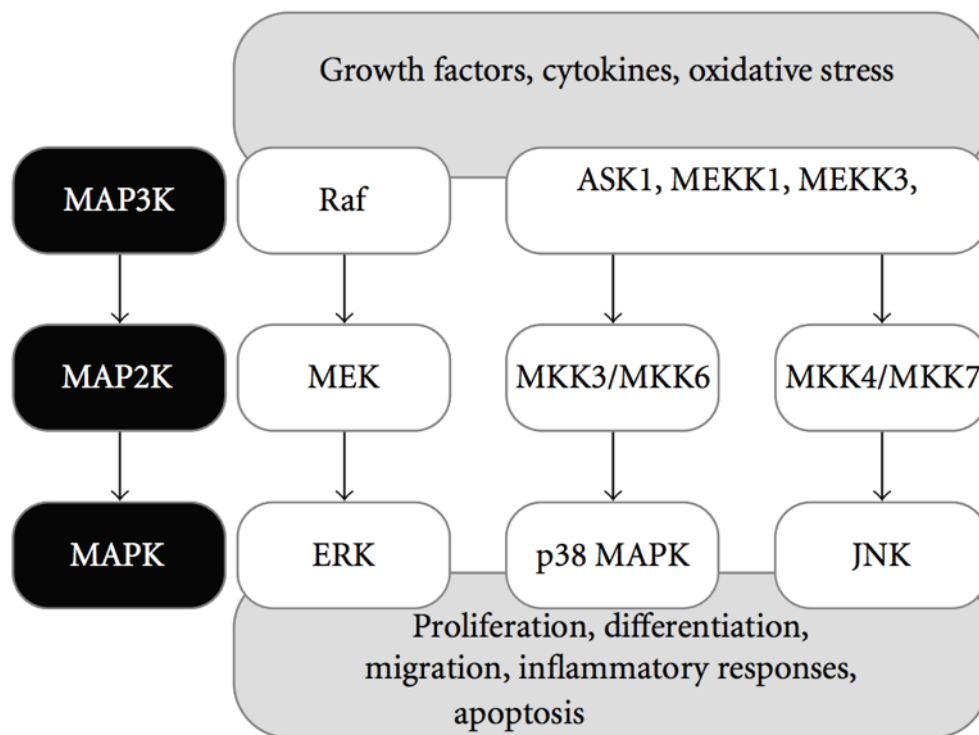


Figure 12. MAPK cascades. MAPK signalling pathways mediate intracellular signalling initiated by extracellular or intracellular stimuli. MAP3Ks phosphorylate MAP2Ks, which in turn phosphorylate MAPKs. Activated MAPKs phosphorylate various substrate proteins (e.g., transcription factors), resulting in regulation of various cellular activities (e.g., proliferation, differentiation, inflammatory responses, and apoptosis). Activation by MAPK signalling cascades is achieved through a series of binary interactions among the kinase. Adapted from Son et al., 2011.

1.3 Oxidative stress in depression

1.3.1 Oxidative stress in clinical studies of depressed patients

Over the last decades elevated biomarkers of OS in MDD have been reported across many studies providing evidence for its contribution to the pathophysiology of depression, and suggesting clinical implications of these findings with regards to novel treatment strategies (Smaga et al., 2015). In order to assess the level of OS in clinical samples different products of oxidation or levels of antioxidants are widely used. These are indirect markers of OS, however due to the very short half-life of ROS this is the only possible way to obtain information on the oxidative state of the subject. Commonly, end products of lipid peroxidation mentioned in section **1.2.3.2**, such as F2-IsoPs, 4-HNE and MDA; of DNA oxidation, such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) mentioned in section **1.2.3.3**; and antioxidative enzymes SOD, catalase (CAT), GSH, glutathione peroxidase and glutathione reductase, are reported in studies examining the rates of OS in depression and response to therapy (Lopresti et al., 2014).

Several meta-analyses showing interest in the oxidative status of depressed patients have been conducted recently. One of these included all studies reporting an association between depression and OS and antioxidant status markers, and found a positive correlation between the illness and the oxidative and antioxidant parameters. (Palta et al., 2014). The heterogeneity among included studies was high: sample sizes varied from 30 to 3000 subjects, there were 12 different OS measures, with MDA and nitric oxide being most frequently reported, and diverse antioxidant status were assessed, with SOD and glutathione peroxidase being the most common. Since an association between depression and OS was found with this variety of measures and diverse samples, this might suggest that it is even a more robust finding. A recent

meta-analysis which focused only on two measures of oxidative damage in depressed patients, including ten studies measuring 8-OHdG and eight studies evaluating F2-isoprostanes, revealed that both OS markers are increased in depressed patients when compared to control groups, indicating that depression is associated with increased oxidative damage to DNA and lipids (Black et al., 2015). Another meta-analysis that looked at studies measuring lipid peroxidation only in depressed patients, consisting of 17 studies, also demonstrated that lipid peroxidation was greater in MDD than in controls and was correlated with depressive symptom severity (Mazereeuw et al., 2015).

Findings from the most recent studies that were not yet included in published meta-analyses are not fully consistent. A cross-sectional and longitudinal study with a sample of 3009 participants revealed an association of depressive symptoms with increased F2-isoprostanes and decreased carotenoids, known to possess antioxidant properties. Although F2-isoprostanes were higher in subjects with depression after adjustment for socio-demographic characteristics, there was no difference after further adjustment for health and lifestyle factors. Additionally, carotenoids were lower in depressed patients independent of all factors, as revealed by regression analyses (Black et al., 2016). Later the same research group published results from another study conducted with a separately recruited smaller group of MDD patients ($n = 2251$), where a different diagnostic tool had been used for MDD assessment, and where oxidative biomarkers had been measured at one time point only. Contrary to previous findings, it was reported that OS was not increased in MDD patients, as no difference in the levels of F2-isoprostanes and 8-OHdG between depressed patients and healthy controls was observed. However, 8-OHdG levels were lower in patients receiving antidepressant therapy than in those with no treatment or controls,

suggesting antioxidant properties of antidepressants (Black et al., 2016). Finally, another recent study that assessed oxidative status in drug naive, first episode, non-smoker major depression patients found that MDA levels were significantly higher and SOD activity significantly lower in depressed patients than healthy controls, with similar CAT activity in both groups (Camkurt et al., 2016).

In addition to human *in vivo* studies, a number of post-mortem studies detected differences in the oxidative status of depressed patients. These include a significant increase in oxidative damage to RNA but not DNA observed in 47 samples of hippocampal tissue (dentate gyrus, CA1, CA3 regions) from patients with MDD (Che et al., 2010) and elevated concentration of SOD in the prefrontal cortex, but not in the hippocampus from seven depressed patients (Michel et al., 2007). Although caution must be taken as the sample size in this study was small, it might suggest that an increase in antioxidant defence biomarkers is due to a compensatory response to OS. A later study identified diminished levels of reduced, oxidized, and total glutathione in 14 samples of post-mortem prefrontal cortex from depressed patients compared to controls (Gawryluk et al., 2011).

1.3.2 Association between increased OS, MDD and other factors: severity of MDD, gender differences and telomere length

In addition to the association between depression and OS status, some studies addressed also some specific issues, such as correlation of the severity of depression with levels of OS, gender-related differences in regards to oxidative status, links to gender-associated MDD vulnerability and correlation between depression and telomere shortening, known to be mainly caused by OS.

Thus, one study demonstrated a direct relationship between the potency of OS and the severity of depression (Yanik et al., 2004). However, later research that hypothesised that patients with a first episode of depression and patients with recurrent major depression would have different levels of oxidative status and immune-inflammatory markers on the level of mRNA reported no differences in the expression of SOD2 gene, coding the enzyme responsible for detoxifying superoxide generated by mitochondrial respiration (Talarowska et al., 2015). Another study that aimed to determine whether any gender-related difference exists concerning OS revealed that women are more susceptible to OS than male. However these gender-based differences do not seem to provide a biochemical basis for the epidemiologic differences in MDD susceptibility between sexes, which indicates that OS is not an independent factor contributing to MDD vulnerability (Wiener et al., 2014). Another cross-sectional study that focused on middle-aged females and researched association between oxidative status and various health parameters such as menopausal status, body composition, cardiovascular characteristics, lifestyle factors (e.g., smoking, exercising) and depressive symptoms, found that out of all parameters being investigated only depression was positively correlated with high levels of urinary 8-OHdG (Hirose et al., 2016).

As mentioned above, there is a high rate of comorbidity between age-related somatic illnesses and early mortality in depressed patients (Maurya et al., 2016), which suggests the notion that these diseases may share the same biological pathways and pathological mechanisms, with OS having a key role (Maes et al., 2011). As such, OS is known to be involved in the pathogenesis of age-related diseases; it is recognised to play a key role in cellular senescence and to be a major mechanism for telomere damage, itself indicative of aging (Houben et al., 2008). Telomeres are

nucleoprotein structures located at the ends of chromosomes with high sensitivity to oxidative damage due to their high content of guanines. Their function is to prevent chromosomal ends from fusion and degradation, and they are subjected to shortening at each cycle of cell division. Senescent cells were found to accumulate oxidative damage to nucleobases and contain 30% more oxidative modified guanine in their DNA and four times as many free 8-OHdG (Valavanidis and Vlachogianni, 2010). 8-OHdG is a particular oxidative transformation of one of the DNA bases that is commonly measured as a marker of OS and have been found increased in various neurodegenerative diseases and depression (Wang et al., 2005; Jorgensen et al., 2013). A recent study showed that patients with depression had more DNA breaks, alkali-labile sites and oxidative DNA damage compared with non-depressed individuals, and that those lesions may be accumulated by impairments of the DNA repair systems (Czarny et al., 2015). In addition, a recent cross-sectional study demonstrated that MDD patients had significantly higher mitochondrial oxidative damage than the comparison group (Chang et al., 2015a).

Several studies demonstrated an exponential correlation between cellular OS levels and telomere shortening rates, suggesting the significant contribution of OS—mediated DNA telomere damage as an important determinant of cellular senescent phenotype (Blackburn, 1991; Kawanishi and Oikawa, 2004; von Zglinicki, 2000). An initial study examining leucocyte telomere length found a significant reduction in their length in depressed patients versus healthy controls, but with no influence due to the applied therapy, the duration of illness or the severity of depression (Hartmann et al., 2010). However, later studies with much larger samples indicated that accelerated aging at the level of leukocyte telomeres was proportional to lifetime exposure to MDD and also inversely correlated with OS, both in depressed subjects as well as in

controls (Verhoeven et al., 2014; Wolkowitz et al., 2011). A recently conducted meta-analysis that summarised all research that has been carried out in this field and included 21040 participants concluded that depression was associated with shorter telomere length (Schutte and Malouff, 2015). Thus, it would be interesting to perform further research on the correlation between the potency of OS and MDD severity, as well as regarding gender susceptibility to OS and MDD, in order to advance in personalised treatment approach. Of great importance is studying factors that might prevent OS-induced telomere shortening in depression. By addressing this issue patients might benefit not only in regards to depression treatment but other conditions comorbid to MDD.

1.3.3 Effects of antidepressant treatments on OS in clinical studies

The involvement of OS in the pathophysiology of depression is further supported by the reduction of OS biomarkers levels in response to antidepressant treatment observed in patients (Lee et al., 2013). A recent meta-analysis that included 29 studies identified elevated levels of MDA accompanied by decreased levels of the antioxidant compounds uric acid and zinc and the antioxidant-enhancing enzyme SOD in MDD patients vs controls before treatment, while there was no change in total nitrites, CAT and glutathione peroxidase. This study demonstrated that different classes of antidepressants, which significantly reduced HAM-D scores, reduced MDA and increased uric acid and zinc levels, so that no distinction was observed in the levels of these markers when compared with healthy controls (Jiménez-Fernández et al., 2015). Another meta-analysis that integrated such parameters as TAC, various enzymatic and non-enzymatic antioxidants, some free radicals and oxidative damage products

included 273 studies and identified increased levels of OS markers and decreased levels of TAC and several antioxidants in depressed patients against healthy controls (Liu et al., 2015). Upon pharmacotherapy of these patients, increased serum uric acid, albumin and vitamin C levels and decreased serum nitrite, red blood cells and serum MDA levels were observed (Liu et al., 2015). A further study published after the meta-analyses measured superoxide and hydroxyl radicals and TAC in the plasma of healthy controls and in 35 first-episode depressed patients before and after receiving a 12-week treatment of sertraline using a very sensitive chemiluminescence method. The results indicate that patients with MDD had significantly lower TAC values at baseline than healthy controls, while there were no significant differences in baseline levels of superoxide and hydroxyl radicals between patients with MDD and control subjects. At the end of the 12-week antidepressant treatment, TAC values were significantly higher and the levels of superoxide radical and hydroxyl radical were significantly lower than baseline values (Chang, et al., 2015). Interestingly, not only pharmacological interventions for depression may result in reduction of OS. It was reported that in 49 MDD subjects with increased level of lipid peroxidation and a decrease in total thiol content before treatment, these parameters were reversed by seven individual weekly sessions of cognitive behavioural therapy (Kaufmann et al., 2015).

However, results are not consistent, as some studies failed to find alterations in oxidative status of MDD patients by antidepressants (Maes et al., 2009b; Sarandol et al., 2007). Discrepancies in findings could be explained mostly by inconsistency in OS biomarkers and antioxidative parameters being used across studies or by treatment-resistant groups being examined. Consistent with this idea, in a recent study when the MDD group was subdivided into those who showed clinical response to

antidepressant therapy and those who did not, only the non-response group showed lower TAC, while the response group showed no significant difference to controls at baseline. After eight weeks of antidepressant treatment, TAC in both the response and non-response groups was similar, and there was no significant difference among the groups (Baek et al., 2016).

Despite the dominant hypothesis that antidepressants restore noradrenergic and serotonergic neurotransmitter systems, a new concept of antidepressant action has been suggested, based on the growing evidence demonstrating the antioxidant effects of these medications (Zafir et al., 2009). Nevertheless, the molecular mechanisms underlying the antioxidative properties of antidepressants are not yet understood. In this thesis the *in vitro* effects of antidepressants preventing oxidative damage in a relevant cell model are examined.

1.3.4 Findings of molecular mechanisms of OS in MDD, coming from animal models of depression

This thesis addresses the lack of relevant *in vitro* studies on OS and MDD and aims at furthering the understanding of potential molecular mechanisms linked to redox alterations in depression. Most of the insights into molecular mechanisms of OS involvement in the pathogenesis of MDD and antioxidative mechanisms of antidepressants come from animal models of depression and therefore it is worth briefly describing them. Overall, these pre-clinical models report lipid and protein peroxidation, DNA damage and a general down-regulation of antioxidant defence in different regions of brain tissues, plasma and serum. Conversely, a reduction of the

oxidative status along with attenuation of depressive-like behaviour is reported upon use of antidepressants (Smaga et al., 2015).

Studies that sought to investigate the molecular mechanisms of these changes in rodents suggested that increased OS in chronic isolation models of depression might occur due to alterations in the primary antioxidant transcription factor Nrf2–Keap1 signalling pathway (Djordjevic et al., 2015; Martín-de-Saavedra et al., 2013; Martín-Hernández et al., 2016) or NADPH oxidase 2 activation in microglia (Schiavone et al., 2009). For example, inhibition by apocynin of the family of NADPH oxidase enzymes (which generates superoxide) prevented the depressive-like behavioural and histopathological oxidative alterations induced by social isolation in rats (Colaianna et al., 2013). Fluoxetine demonstrated to recover the activity levels of the antioxidative SOD, CAT and GSH in the rat brain after restraint stress and partially reversed the increased generation of ROS in peripheral blood cells obtained from restraint-stressed mice (Novio et al., 2011; Zafir et al., 2009). In addition, fluoxetine reversed the decreased expression of Nrf2 and its downstream genes in the cortex and hippocampus in a mouse model of depression induced by glucocorticoids to mimic the effects of chronic stress and induce an anxiety/depression-like phenotype in mice. (Mendez-David et al., 2015). The long-term administration of venlafaxine, that has been also studied in this thesis, protected against stress-induced oxidative lipid and DNA damage in male mice (Abdel-Wahab and Salama, 2011). Additionally, a single dose of the novel promising fast-acting antidepressant compound ketamine, which was also under investigation in my thesis, induced long-term antidepressant effects and reversed the protein oxidative damage and lipid peroxidation produced in adult rats following maternal deprivation (Réus et al., 2015). However, pro-oxidative effects of ketamine have also been reported, including increased hydroxyl radical

production and lipid peroxidation, and reduced activity of SOD and CAT in the rodent brain (da Silva et al., 2010; de Oliveira et al., 2009; Zuo et al., 2007). More research is warranted.

1.3.5 Antioxidant compounds with antidepressant properties

Since antidepressants have been demonstrated to restore altered redox homeostasis in MDD patients and animal models of depression, studies have examined whether antioxidant compounds might exhibit antidepressant properties (Xu et al., 2014). The literature on neuroprotective and antidepressant properties of various exogenous antioxidants ranging from rare herbs (Bahramsoltani et al., 2015) to pharmacological agents that have been widely used in medicine for a long time like NAC is enormous. It is not yet well-defined what molecular mechanisms of antioxidants lead to antidepressant effects, but it is determined that in a biological context, antioxidant compounds protect against oxidative damage by these main mechanisms: preventing ROS formation by sequestering transition metal ions into complexes, scavenging or quenching free radicals and other reactive species, breaking chain reactions initiated by free radicals and inducing antioxidants and repair systems (Scapagnini et al., 2012). Therefore, my work sought to investigate potential antioxidative mechanisms of various antidepressant compounds, also using NAC as one of the conventional antioxidants with antidepressant properties as a positive control.

NAC, a thiol compound that can act as a direct ROS scavenger or provide cysteine for glutathione production, has been used to treat psychiatric and neurological disorders over the past decade. Although conclusions are mixed, NAC efficacy has been demonstrated in the treatment of Alzheimer's disease, autism, schizophrenia, bipolar

disorder and addictions (Deepmala et al., 2015). A recent meta-analysis that included 574 participants found that MDD subjects receiving NAC improved depressive symptoms as assessed by Montgomery-Asberg Depression Rating Scale and HAM-D when compared to placebo, had better depressive symptoms scores on the Clinical Global Impressions-Severity of Illness scale at follow-up and showed better global functionality than subjects on placebo (Fernandes et al., 2016). Animal studies that determined antidepressant properties of NAC attributed its effect to direct antioxidant characteristics (Ferreira et al., 2008; Smaga et al., 2012), capability to inhibit inflammation (Beloosesky et al., 2012; Möller et al., 2013), potentiation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors and as a result indirect modulation of noradrenaline and serotonin pathways (Costa-Campos et al., 2013; Linck et al., 2012) and direct modulation of cortico-striatal monoamines (Möller et al., 2013). The evidence of NAC efficacy in MDD patients is however limited. A double-blind, randomized, placebo-controlled trial conducted over 12 weeks revealed positive effects of NAC added to conventional antidepressants, observed only 4 weeks after discontinuation of the treatment, and in the group with more severe depression or those bipolar patients who were in the depressive phase (Berk et al., 2008).

Other extensively studied antioxidant compounds with antidepressant potential are flavonoids (Gong et al., 2014; Guan and Liu, 2016; Herrera-Ruiz et al., 2011), ascorbic acid (Binfaré et al., 2009; Khajehnasiri et al., 2016; Moretti et al., 2012), α -tocopherol (Lobato et al., 2010; Sepehrmanesh et al., 2016), coenzyme Q10 (Aboul-Fotouh, 2013), different polyphenols, such as curcumin (Seo et al., 2015) and resveratrol (Ge et al., 2013). For example curcumin, a polyphenolic substance, reduces OS through activation of heterodimers of Nrf2, leading to induction of the

antioxidant defensive genes and inhibition of MAO-A and MAO-B. In addition, curcumin modulates the levels of noradrenaline, dopamine and serotonin in the brain and decreases inflammation. (Kaufmann et al., 2016; Scapagnini et al., 2012). These findings provide a convincing rationale for studying molecular mechanisms of antioxidant compounds and investigating their effects beyond the re-establishing of redox balance.

1.3.6 Omega-3 fatty acids as potential antidepressants with antioxidative properties

It is well recognised that omega-3 PUFAs are essential for nervous system activity, cognitive development, memory-related learning, neuroplasticity of nerve membranes, synaptogenesis and synaptic transmission (Mazza et al., 2007). The omega-3 PUFAs are derived from fish and some plants and they cannot be endogenously synthesized in humans. Although there are numerous PUFAs, the omega-3 fatty acids EPA (eicosapentaenoic acid, 20:5 ω 3) and DHA (docosahexaenoic acid, 22:6 ω 3) have been found to be particularly important in brain function, along with their precursor molecule ALA (α -linolenic acid, 18:3 ω 3) (Beltz et al., 2007). EPA and DHA are incorporated into neuronal phospholipids, and the fatty acid composition determines the biophysical properties of neuronal membranes and influences neurotransmission (Mazza et al., 2007). PUFAs have been shown to inhibit various aspects of inflammation, known to contribute to shifts in redox state, in both animals and humans (Calder, 2015; Dylla, 2015; Rapaport et al., 2016). In humans, positive correlations have been found between higher levels of EPA and DHA in red blood cells and larger total brain volume and hippocampal volume, which

is consistently reported to be decreased in MDD (Pottala et al., 2014). A recent review that analysed 24 studies on the effect of PUFA intake on brain function and structure indicated that EPA and DHA intake may be associated with increased functional activation of the prefrontal cortex in children, and greater grey matter volume and white matter integrity during aging. However, the study found limited evidence to support that omega-3 PUFA supplementation is beneficial in MDD (Bos et al., 2016). Although clinical trials give conflicting results on antidepressant properties of PUFAs, a recent meta-analysis demonstrated that PUFA preparations with EPA accounting for greater than 60% of their composition were effective against depression, while those with less were ineffective (Sublette and Ellis, 2011). Another meta-analysis comprised of 14 studies demonstrated lower levels of EPA, DHA and total omega-3 PUFAs in depressed patients suggesting that fish oils contribute to the pathophysiology of depression (Lin et al., 2010). Later it was identified that chronic or severe MDD cognitive decline is associated with lower EPA concentrations and higher AA/EPA ratios in erythrocyte membranes (Chiu et al., 2012). Interestingly, significant improvement in cognitive performance, after dietary PUFAs intake, particularly DHA treatment, was observed in children with low literacy ability and malnourishment, as well as in older adults with age-related and/or mild cognitive decline (Stonehouse, 2014). The most recent evidence indicates that MDD responds to EPA or EPA + DHA treatments, differentially or synergistically, with the most effective effects observed with higher doses of EPA combined with lower doses of DHA (Song et al., 2016). The combination of EPA with SSRIs had been shown to ameliorate MDD better than antidepressant monotherapy, suggesting that there are synergetic effects between fish oils and antidepressant treatment (Gertsik et al., 2012). Additionally, recent clinical study demonstrated that patient's high baseline levels of

EPA and DHA predict favourable depression outcomes in patients receiving omega-3 supplements together with conventional antidepressant (Carney et al., 2016). A very recent study, where an inverse association between the omega-3 index and depressive symptoms was observed among participants with elevated OS biomarkers, linked together the notion that PUFAs possess both antioxidative and antidepressant properties (Bigornia et al., 2016). These data suggest that the OS status may identify those who might benefit from fish oils consumption to improve depressive symptoms. Additionally, *in vitro* and *in vivo* research suggest that omega-3 PUFAs and EPA in particular exert antidepressant-like effects by modulating monoamine neurotransmission, inflammatory responses and neurogenesis (Song et al., 2016). Deteriorated serotonergic, noradrenalinergic, dopaminergic neurotransmission and hypercortisolemia, that are often found in depressed patients have been related to omega-3 PUFA deficiency in rats, suggesting that supplementation with omega-3 PUFAs may restore the monoamine neurotransmission and normalize HPA function in MDD (Kodas et al., 2004; Zimmer et al., 2002). This is supported by the observation that an omega-3 PUFA enriched diet promotes hippocampal plasticity and BDNF levels in mice (Venna et al., 2009). Additionally, DHA reverses the anti-neurogenic effect of LPS-activated microglia in mouse neural progenitor cells (Antonietta Ajmone-Cat et al., 2012) and increases neurite outgrowth of immature neurons in embryonic hippocampal cells (Cao et al., 2009). DHA-deficient embryonic rat brains show decreased neurogenesis (Coti Bertrand et al., 2006), while transgenic mice with high levels of endogenous DHA demonstrate increased levels of neurogenesis (He et al., 2009). Also, DHA partially alleviates age-related reductions in levels of neurogenesis in rats (Dyall et al., 2010). Although PUFAs are known to

be linked to neurogenesis, the mechanism behind its role has yet to be elucidated (Crupi et al., 2013).

Given the antidepressant and antioxidative properties of EPA and DHA, in this thesis I sought to investigate the capability of these PUFAs to prevent the exogenously induced OS in my *in vitro* model and some possible underlying mechanisms.

1.4 Human hippocampal progenitor cells as a research tool to study the role of OS in depression and effects of antidepressant compounds

Previous work aimed at understanding the role of OS in the pathogenesis of human depression has been limited to examinations of peripheral samples or post-mortem brain tissues. In contrast to the plenty of data available from animal studies, no *in vitro* studies have looked at the role of OS or the antioxidant properties of antidepressants in human brain cells. Moreover, despite a plethora of evidence available from clinical trials and animal models suggesting that treatment with antidepressants and antioxidants can reduce OS and ameliorate depressive-like behaviours, a poor correlation between *in vitro* and *in vivo* antioxidant properties of these compounds has been observed. Therefore, both approaches, while useful, do not allow a species-specific and cell-specific understanding of the pathological pathways, that would allow a better translation to clinical practice. The human hippocampal progenitor cell line HPC0A07/03C used in this thesis provides a relevant experimental platform to study the role of OS, including underlying molecular mechanisms. These cells exhibit the same characteristics as adult hippocampal progenitor cells: they express specific progenitor markers, such as GFAP, nestin and SOX2, they proliferate indefinitely, and they have the potential to develop into the

main cell types of the human central nervous system: neurons, astrocytes and oligodendrocytes, recapitulating human neurogenesis. This cell line has been successfully validated as a model of "depression in a dish". It has been used to study other domains of the pathogenesis of the disorder, including the role of stress and inflammation, and the ability of monoaminergic antidepressants and PUFAs to modify these domains (Anacker et al., 2013a, 2013b, 2011; Horowitz et al., 2015; Zunszain et al., 2012a). Here I present the first *in vitro* study that models OS in these cells, examining its role in neurogenesis, investigating molecular changes and the ability of antidepressant compounds to prevent the detrimental oxidative damage.

1.5 Aims and hypothesis of the study

This thesis aims to evaluate the cellular and molecular effects of OS on human hippocampal progenitor cells, as well as the ability of antidepressant compounds to prevent detrimental changes caused by oxidative damage. Specifically, this thesis aims to address the following:

Aim 1. To establish a controlled and robust model of OS in the human hippocampal progenitor cells;

Aim 2. To study how neurogenesis is affected in the cells upon the chosen OS treatment, by looking at proliferation and differentiation markers and identifying potential signalling pathways underlining changes observed;

Aim 3. To test the hypothesis that the chosen OS treatment causes activation of the pleiotropic transcription factors NF- κ B and Nrf2, and to evaluate whether this leads to downstream inflammation and up-regulation of antioxidative stress genes.

Aim 4. To compare the effects of antidepressants and fish oils versus antioxidants on the prevention of oxidative damage inflicted by the chosen OS treatment.

2 Materials and Methods

2.1 Study design

This thesis examines the damaging effects of OS, known to contribute to the pathogenesis of depression, on human hippocampal progenitor cells and the ability of different compounds with antidepressant action (including a selective serotonin reuptake inhibitor, a serotonin–noradrenaline reuptake inhibitor, an *N*-methyl-D-aspartate (NMDA) receptor antagonist and two polyunsaturated fatty acids) to prevent the detrimental changes inflicted by the OS insult. Additionally, the effects of OS on neurogenesis, that is considered to be impaired in depressed patients, were researched in this project. In order to investigate the cellular and molecular changes caused by exogenously-induced OS, a human embryonic hippocampal progenitor cell model (HPC0A07/03C, ReNeuron, UK) that is capable of neurogenesis and is of particular relevance to depression has been utilised. OS was induced by treating the cells with a range of concentrations of 70% aqueous solution of *tert*-butyl hydroperoxide (*t*-BHP, Sigma), which is a membrane permeable oxidant that can be easily metabolized and cause a variety of changes in biological and biochemical systems. *t*-BHP is an organic hydroperoxide that has been previously used successfully as a compound to generate OS damage in various *in vitro* models, including rat primary neuronal cultures (Shimmyo et al., 2008) and human astrocytes (Steinbrenner et al., 2006), and it was chosen due to its higher stability than H₂O₂, another commonly used peroxide to induce OS *in vitro* (Liu et al., 2014). HPC0A07/03C cells were expanded in culture and subsequently used for a variety of assays described in this thesis upon induction of redox shifts in the system. The underlying changes in cell viability, lipid peroxidation, immunoprotein secretion, transcription factor activation, proliferation,

differentiation and gene expression caused by OS were studied with a range of cellular and molecular biology techniques. Cell viability was determined using the MTS assay (Promega) in live cells. To assess the level of lipid peroxidation I used the TBARS assay (R&D), measuring the amount of MDA and other thiobarbituric acid substances in the supernatant. Immunocytochemistry was conducted to detect changes in proliferation and differentiation caused by *t*-BHP using specific antibodies for proliferation markers and for immature and mature neurons. Changes in the levels of apoptotic marker Cleaved Caspase-3 (CC3) and NF- κ B p65 subunit nuclear translocation, indicative of its activation, were also assessed with immunocytochemistry using relevant antibodies. All images were analysed using an automated platform for cell imaging the CellInsight* NXT High Content Screening Platform (ThermoScientific). Nrf2 nuclear translocation was measured using a commercially available ELISA kit (Active Motif) using nuclear protein lysates. Kynurenine metabolites were measured in the cell culture supernatant by high performance liquid chromatography and mass spectrometry. Cytokines expression was detected using commercially available kits using ELISA and Luminex technology. Levels of gene expression were determined from whole cell RNA by quantitative real-time PCR. For analysis of protein expression whole cell protein lysates were obtained and subjected to electrochemiluminescence assay (Meso Scale Discovery). All techniques, shown in **Figure 13**, will be described in further detail below.

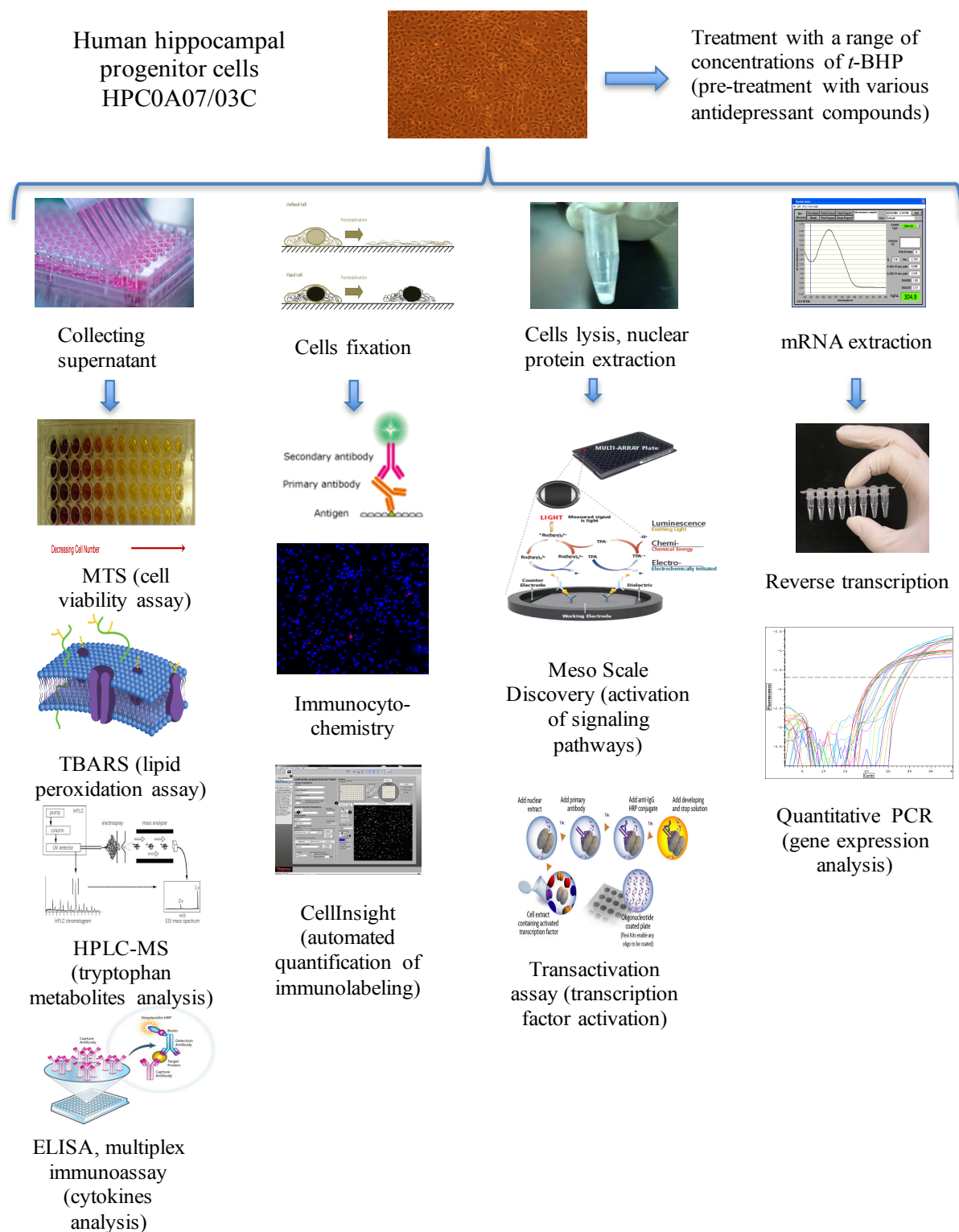


Figure 13. Overview of experimental techniques employed in this PhD thesis. All techniques will be described in detail below.

2.2 Human hippocampal progenitor cells and culture

All experiments were performed using the human embryonic hippocampal progenitor cell line **HPC0A07/03C** (provided by ReNeuron Ltd., Surrey, UK). This is a multipotent progenitor cell line that was conditionally immortalized. Multipotent stem cells have the same basic features of all stem cells, they are unspecialized cells that have the ability to self-renew for long periods of time and to differentiate into specialized cells with specific functions. In contrast to totipotent or pluripotent stem cells, multipotent stem cells are limited in their ability to differentiate, meaning that multipotent hippocampal progenitor cells can give rise to neural cells and glia, but not to other cell types. HPC0A07/03C cells therefore have the potential to proliferate indefinitely before they differentiate into a target cell (neuron, astrocyte or oligodendrocyte), depending on which cell culture condition is applied (**Figure 14**).

HPC0A07/03C cells were generated by ReNeuron Ltd. as described previously (Pollock et al., 2006). Briefly, primary cells were isolated from the hippocampal region of a 12-week-old female foetus and transfected with the c-myc-ERTM gene. The c-myc-ERTM technology was developed by Littlewood and colleagues and is based on a fusion protein comprising the growth promoting gene c-myc and the genetically modified murine oestrogen receptor (ER) G525R (Danielian et al., 1993; Littlewood et al., 1995). The mutant oestrogen receptor is exclusively responsive to the synthetic steroid 4-hydroxytamoxifen (4-OHT) and will activate the c-myc gene only in the presence of 4-OHT (Danielian et al., 1993). HPC0A07/03C cells containing the c-myc-ERTM thus proliferate indefinitely in the presence of 4-OHT, the basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), whereas proliferation is ceased whenever 4-OHT, bFGF and EGF are removed from the cell culture media (Littlewood et al., 1995; Pollock et al., 2006).

Cells were cultured as described by Johansson et al. (2008) with minor modifications. Working stock cultures of 2×10^6 cells/ vial, passaged 11 times after they were generated (p11), were kept in 10% DMSO solution in liquid nitrogen at -120°C . Upon removal from the liquid nitrogen tank, cells were quickly thawed in a water bath at 37°C under constant agitation, transferred to a microbiological safety cabinet and resuspended in 10 ml of fresh Reduced Modified Media (RMM) consisting of Dulbecco's Modified Eagle's Media/F12 (DMEM:F12, Invitrogen, Paisley, UK) supplemented with 0.03% human albumin (Baxter Healthcare, Compton, UK), 100 $\mu\text{g/ml}$ human apo-transferrin, 16.2 $\mu\text{g/ml}$ human putrescine DiHCl , 5 $\mu\text{g/ml}$ human rec. insulin, 60 ng/ml progesterone, 2 mM L-glutamine and 40 ng/ml sodium selenite. To maintain proliferation, 10 ng/ml human bFGF, 20 ng/ml human EGF and 100 nM 4-OHT were added (**Table 3**). To induce differentiation, cells were cultured in RMM without bFGF, EGF or 4-OHT (later will be referred as RMM---) (**Table 4**). Cells were centrifuged at 900 rpm for 5min, media supernatant was aspirated, and cells were resuspended in fresh media to remove any residual DMSO. Cells were counted using a standard Neubauer hemocytometer and 1.2×10^6 cells were plated on laminin pre-coated 75cm^2 tissue culture flasks (Nunc) in 12 ml RMM. During normal expansion in a humidified cell culture incubator at 37°C and 5% CO_2 , HPC0A07/03C cells proliferate with a doubling time of 72 hours (Johansson et al., 2008). Cells were thus passaged every 72 hours at approximately 80% confluence. For passaging, cell culture media was aspirated and cells were incubated in 3 ml pre-warmed Accutase (Sigma) for 5 min at 37°C to detach cells from the flask. The bottom of the flask was then washed with 6 ml pre-warmed RMM (with a 1:2 ratio Accutase:RMM) and the cell suspension was collected in 15 ml centrifuge tubes (Corning). Cells were

centrifuged at 900rpm for 5min, supernatant was aspirated, cells were resuspended in RMM and re-seeded at a cell density of 1.2×10^6 cells per 75cm² tissue culture flask.

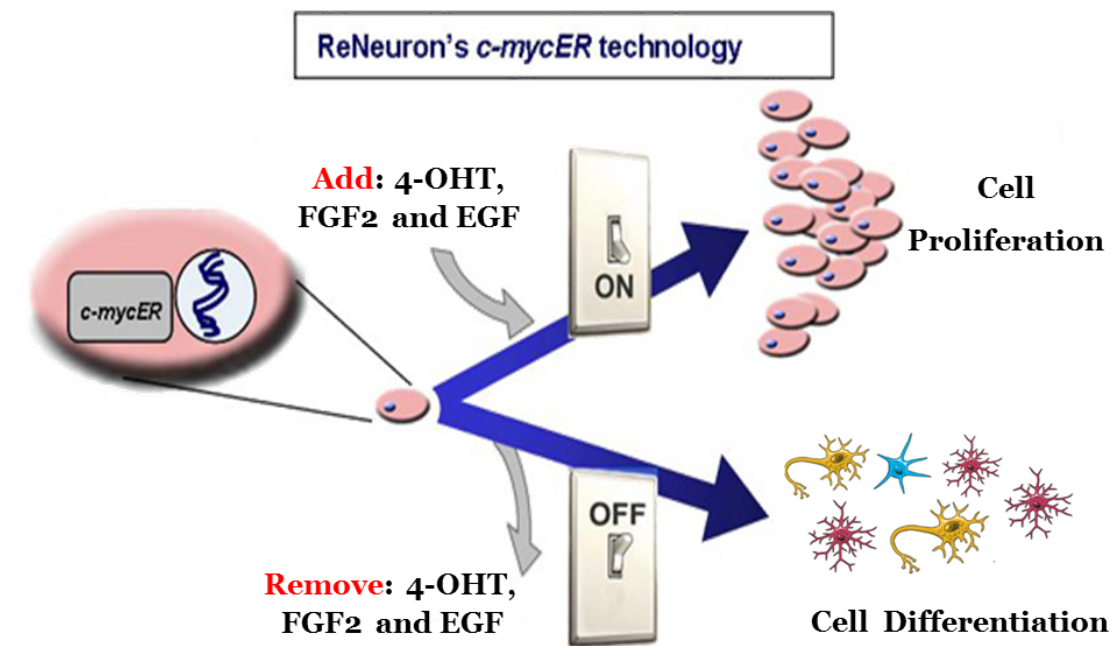


Figure 14. Schematic of ReNeuron's HPC0A07/03C cells with c-myc-ERTM technology. Human embryonic hippocampal progenitor cells stably expressing the c-myc-ER transgene proliferate in the presence of the growth factor EGF and bFGF, and the synthetic compound 4-OHT which activates the c-myc-ER transgene. Upon removal of EGF, bFGF and 4-OHT, HPC0A07/03C cells cease to proliferate and start to differentiate into neurons, astrocytes and oligodendrocytes. Picture source: www.reneuron.com

RMM proliferation media (with growth factors and 4-hydroxytamoxifen)		
Compound	Concentration	Supplier
DMEM:F12	1:1	Invitrogen
human albumin solution	0.03%	Baxter Healthcare, Ltd
human apo-transferrin	100 µg/ml	Scipac
human putrescine DiHCl	16.2 µg/ml	Sigma Aldrich
human rec. insulin	5 µg/ml	Sigma Aldrich
progesterone	60 ng/ml	Sigma Aldrich
L-glutamine	2 mM	Sigma Aldrich
sodium selenite	40 ng/ml	Sigma Aldrich
human bFGF	10 ng/ml	Peptotech EC
human EGF	20 ng/ml	Peptotech EC
4-OHT	100nM	Sigma Aldrich

Table 3. Components added to DMEM for proliferation media. (DiHCl, dihydrochloride; rec, recombinant; FGF, fibroblast growth factor; EGF, epidermal growth factor; 4-OHT, 4-hydroxytamoxifen)

RMM (-) differentiation media (<u>without</u> growth factors and 4-hydroxytamoxifen)		
Compound	Concentration	Supplier
DMEM:F12	1:1	Invitrogen
human albumin solution	0.03%	Baxter Healthcare, Ltd
human apo-transferrin	100 µg/ml	Scipac
human putrescine DiHCl	16.2 µg/ml	Sigma Aldrich
human rec. insulin	5 µg/ml	Sigma Aldrich
progesterone	60 ng/ml	Sigma Aldrich
L-glutamine	2 mM	Sigma Aldrich
sodium selenite	40 ng/ml	Sigma Aldrich

Table 4. Components added to DMEM for differentiation media. (DiHCl, dihydrochloride; rec, recombinant)

2.3 Experimental design

2.3.1 Identification of the working doses of *t*-BHP

The first aim of this thesis was to establish a robust model of OS in human hippocampal progenitor cells using *tert*-butyl hydroperoxide (*t*-BHP). Therefore, it was necessary to identify the optimal doses of *t*-BHP and the duration of the treatment that inflicted a significant oxidative damage to the cells. At the same time the purpose of this experiment was to confirm that there was a sufficient amount of viable cells left after being exposed to different concentrations of *t*-BHP in order to follow up the cells fate.

The optimal dose and duration of treatment of *t*-BHP required to provoke a robust OS insult was first determined by examining multiple doses and treatment periods. Cells were plated in 96 well plates (Nunc) at a density of 1.5×10^4 cells per well (**Table 6**) in 100 μ L of RMM in the presence of growth factors and allowed to firmly attach overnight. Subsequently, cells were initially treated with *t*-BHP at a dose of 1 μ M, 2.5 μ M, 5 μ M, 7.5 μ M, 10 μ M, 25 μ M, 50 μ M, 75 μ M, 100 μ M, and 1mM for 24 hours before performing a cell viability assay (**Figure 15a**). Each *t*-BHP concentration represents a separate condition in all experiments described. Following the results of this experiment the range of *t*-BHP concentrations was changed and narrowed down to 1 μ M, 10 μ M, 25 μ M, 50 μ M, 100 μ M, 200 μ M, 500 μ M, and 1mM. Due to dramatic difference in cell viability between close concentrations, 10 μ M and 25 μ M of *t*-BHP, treatment time was reduced to 1 hour, after which fresh media was added, and cells were cultured for the following 23 hours. After 24 hours in total cell viability assay was performed (**Figure 15b**).

2.3.2 Assays with antidepressant compounds

To assess the capability of antidepressants (previously reported to possess antioxidative effects *in vivo*) and omega-3 fatty acids (known to have antidepressant properties) to prevent oxidative damage inflicted by *t*-BHP in human neural progenitor cells the assay described below was conducted. Antioxidants were also used in this assay as a positive control. **Table 5** briefly outlines compounds under investigation and pharmacological class of the drugs.

Cells were plated in 96 well plates (Nunc) at a density of 1.5×10^4 cells per well (**Table 6**) in 100 μ L of RMM with growth factors and allowed to firmly attach overnight. Cells were then pre-treated with each of the compounds in two different concentrations for 1 hour or 24 hours. After that cells were co-treated with the compounds under investigation and 100 μ M or 500 μ M of *t*-BHP for 1 hour followed by 23 hours culturing in fresh media. Subsequently, cell viability assay was performed (**Figure 15c**). This paradigm was used for all experiments, unless otherwise stated.

Treatment doses for antidepressants and omega-3 fatty acids were informed by previous *in vitro* research in our lab with this particular cell line (Anacker et al., 2011; Horowitz et al., 2015), as well as therapeutic levels where known (Schulz et al., 2012) before performing viability assays in our cells. All treatments had the same vehicle (including 0.01% DMSO and 0.1% ethanol) to exclude the possibility of any differences observed being the consequence of differing concentrations of solvents.

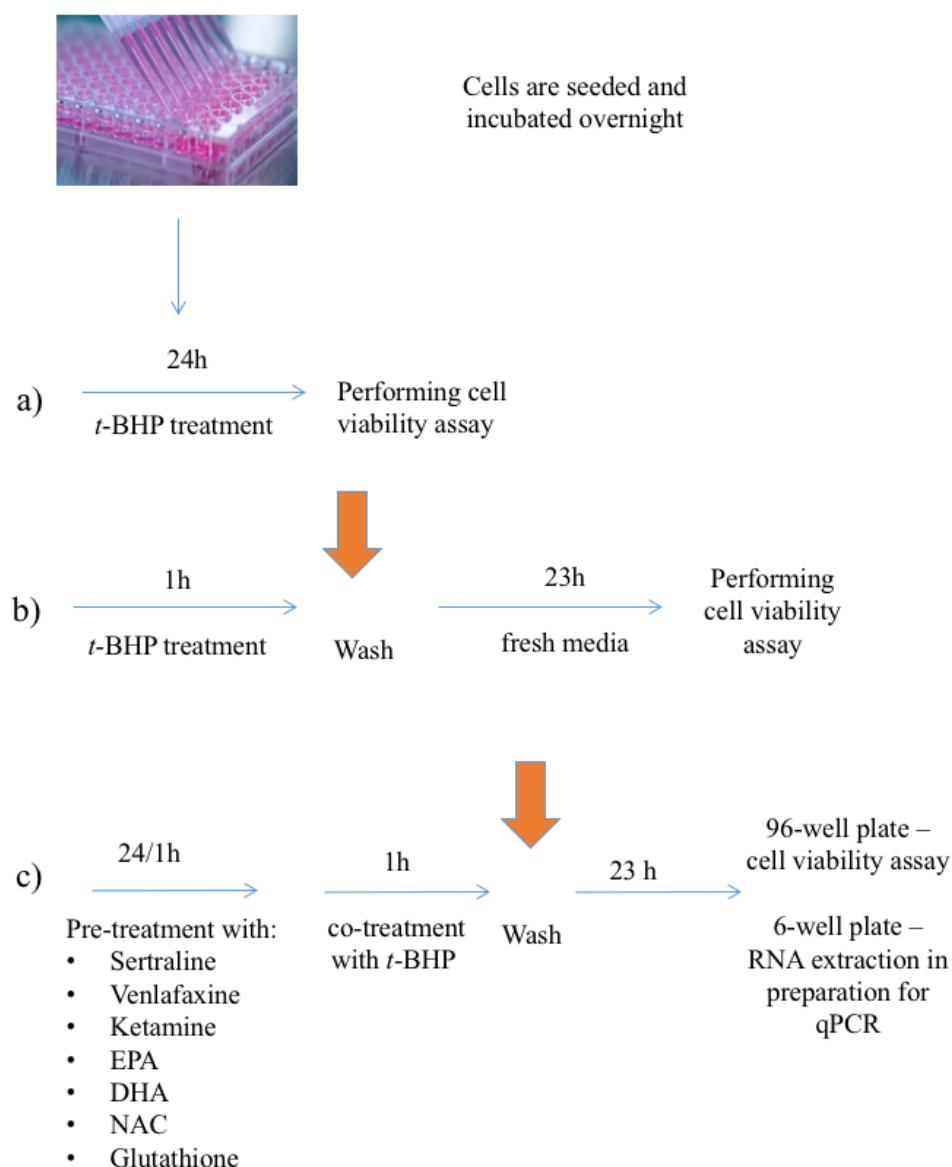


Figure 15. Experimental design. Cells were plated in 96 well plates and allowed to firmly attach overnight. The next day **a)** cells were treated with *t*-BHP for 24 hours and followed by the cell viability, **b)** cells were treated with *t*-BHP for 1 hour and then cells were cultured in fresh media for 23 hours followed by the cell viability assay, **c)** cells were pre-treated with each of the compounds for 1 or 24 hours. After that cells have been co-treated with the compounds under investigation and *t*-BHP for 1 hour followed by 23 hours culturing in fresh media. Subsequently, cell viability assay was performed.

Compound	Pharmacological class
Sertraline hydrochloride	Selective serotonin reuptake inhibitor (SSRI)
Venlafaxine hydrochloride	Serotonin-norepinephrine reuptake inhibitor (SNRI)
Ketamine hydrochloride	N-methyl-D-aspartate (NMDA) receptor antagonist
Eicosapentaenoic acid (EPA)	Omega-3 fatty acid
Docosahexaenoic acid (DHA)	Omega-3 fatty acid
<i>N</i> -Acetyl-L-cysteine (NAC)	<i>N</i> -acetyl derivative of naturally occurring amino acid L-cysteine – a precursor of the biological antioxidant glutathione
Glutathione reduced ethyl ester	Membrane/lipid permeable derivative of biological antioxidant glutathione

Table 5. Comounds under investigation in this thesis.

2.3.3 Proliferation assay

To assess whether the oxidative insults caused by *t*-BHP lead to changes in neuronal proliferation, progenitor cells were plated on 96-well plates (Nunc) at a density of 1.2×10^4 cells per well in 100 μ l RMM media (**Table 6**). Cells were cultured in the presence of EGF, bFGF and 4-OHT for 24 hours prior, followed by 72 hours experimental condition (1 μ M, 10 μ M, 25 μ M, 50 μ M, 100 μ M of *t*-BHP) also in the presence of growth factors (**Figure 16a**). At the end of the total incubation time (72 hours), cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Cells were washed three times in PBS and stored at 4°C in PBS containing 0.2% sodium azide in preparation for immunocytochemistry.

2.3.4 Differentiation assay

As described above for proliferation, to assess whether the oxidative insults caused by *t*-BHP leads to change in neuronal differentiation, progenitor cells were plated on 96 well plates (Nunc) at a density of 1.2×10^4 cells per well in 100 μ l RMM media (**Table 6**). Cells were cultured in the presence of EGF, bFGF and 4-OHT for 24 hours prior, followed by 72 hours incubation experimental condition (1 μ M, 10 μ M, 25 μ M, 50 μ M, 100 μ M of *t*-BHP). After this initial proliferation phase, cells were washed twice for 15 min in RMM (---) media, and then cultured in RMM (---) media with the same concentrations of *t*-BHP for subsequent 7 days. At the end of the total incubation time (10 days) cells were fixed with 4% PFA for 20 min at room-temperature (**Figure 16b**). Cells were washed three times in PBS and stored at 4°C in PBS containing 0.2% sodium azide before further processing by immunocytochemistry. This experimental paradigm was also utilised in 6-well plates

so that protein could be extracted to investigate the signalling pathways underlying changes on neurogenesis.



Cells are seeded and incubated overnight in a 96-well or 6 well plate

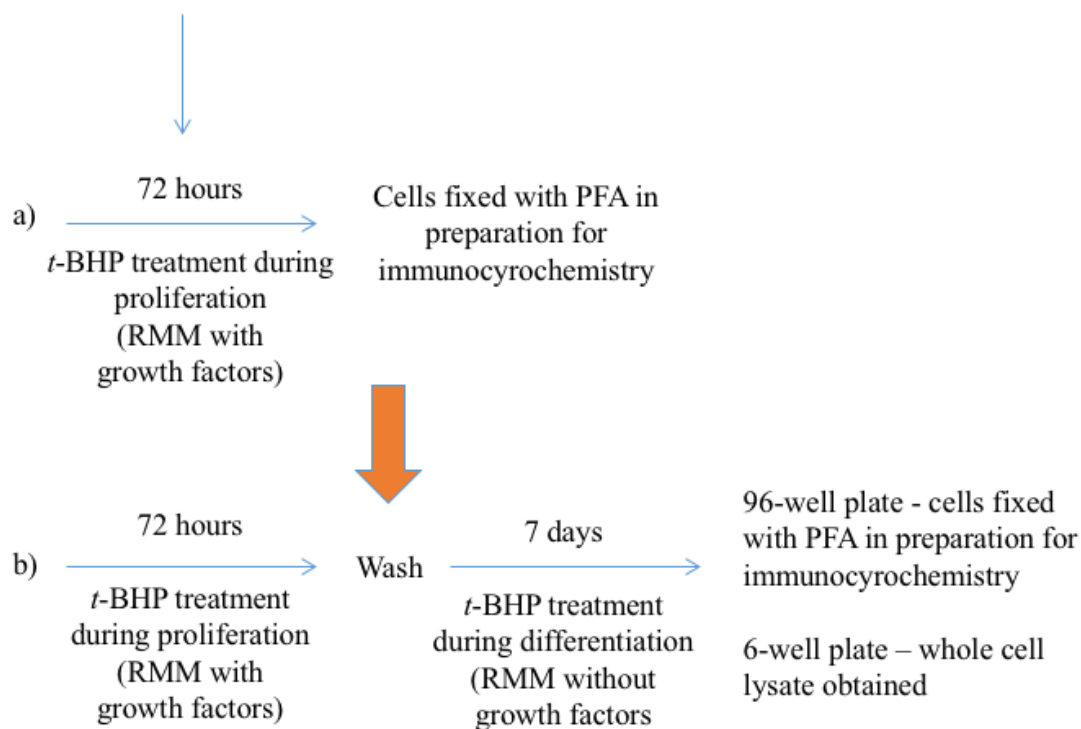


Figure 16. Experimental design for the cell viability assay. Cells were plated in 96 or 6-well plates and allowed to firmly attach overnight. The next day **a)** cells were treated with *t*-BHP for 72 hours in proliferation media and fixed after the incubation time; **b)** cells were treated with *t*-BHP for 72 hours in proliferation media, then washed with warm DMEM and treated with *t*-BHP for the next 7 days. After 10 days in total cells from a 96-well plate were fixed in preparation for immunostaining and from a 6-well plate whole cell lysate was obtained.

Experiment	Plate	Number of cells used
MTS assay	96-well plate	15 000
Immunocytochemistry	96-well plate	15 000 12 000 for 3+7 assay
ELISA, Luminex	96-well plate	15 000
HPLC-MS	96-well plate	15 000
Electrochemiluminescence	6-well plate	350 000
TBARS assay	6-well plate	350 000
qPCR	6-well plate	350 000
TransAM	75cm ² flask	3 000 000

Table 6. Plates and number of cells used in the experiment according to the protocol requirements of the assay.

2.4 Cell viability assessment by MTS assay

Cells were plated in 96 well plates (Nunc) at a density of 1.5×10^4 cells per well in 100 μ L of RMM and allowed to firmly attach overnight (**Table 6**). Depending on the experiment being conducted cells were then either treated directly with *t*-BHP or pre-treated with each of the compounds under investigation followed by *t*-BHP co-treatment (**Figure 15**). Subsequently cell viability assay was performed. Four technical replicates per condition were prepared and at least three independent experiments were conducted on three independent cultures.

To determine cell viability on the culture plate the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was used. This is a colorimetric method that allows to estimate the amount of viable cells in the condition. The kit contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) for enhancing chemical stability. PES allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound is bio-reduced by cells into a coloured formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells.

The procedure was conducted according to the manufacturer's instructions. Briefly, assays were performed by adding 40 μ L of the CellTiter 96® AQueous One Solution Reagent per well per 200 μ L of media directly to culture wells. Cells were incubated for 3 hours in the presence of the assay reagent and then the absorbance was recorded at 490 nm with a using a DTX 880 Multimode Detector (Beckman-Coulter). The quantity of formazan product as measured by absorbance at 490 nm is directly

proportional to the number of living cells in culture after subtraction of the background.

2.5 Lipid peroxidation assessment by TBARS assay

Thiobarbituric acid-reactive substances (TBARS) assay is the most commonly used method for measuring lipid peroxidation, including MDA and other thiobarbituric acid-reactive substances, and was also employed in this research project. Cells were plated in 6-well plates (Nunc) at a density of 3.5×10^5 cells per well in 2ml of RMM and allowed to firmly attach overnight (**Table 6**). Based on previous experiments where the working doses of *t*-BHP were identified, cells were treated with 50µM 100µM, 200µM 500µM and 1mM for 1 hour. Then the supernatant was collected and immediately stored at -80°C for the later lipid peroxidation assay performance. Four independent experiments were conducted on four independent cultures, and each sample was tested in duplicate.

In order to identify the level of lipid peroxidation in the cells exposed to a range of concentrations of *t*-BHP the Thiobarbituric Acid Reactive Substances (TBARS) Parameter Assay Kit (TBARS, R&D) was used. The oxidative degradation of lipids by ROS results in the formation of highly reactive and unstable lipid peroxides. Decomposition of lipid peroxides in turn results in the formation of TBARS, including MDA. In the presence of heat and acid, MDA reacts with 2-thiobarbituric acid (TBA) and produces a coloured end product that is measured with a 96 plate reader at 532 nm (**Figure 17**). The intensity of the colour corresponds to the level of lipid peroxidation in the sample.

The procedure was performed according to the manufacturer's instructions. Briefly, in order to clarify the samples from precipitating interfering proteins and other substances, prior to the assay performance 300 μ L of the sample was treated with 300 μ L of TBARS Acid Reagent in a microcentrifuge tube. Then samples were mixed, incubated for 15 minutes at room temperature and centrifuged at 14,000 x g for 4 minutes. Supernatants were retained and used in the assay. Afterwards, 150 μ L of standards and samples were added to each well followed by addition of 75 μ L of TBA Reagent. The optical density of each well was pre-read using a using a DTX 880 Multimode Detector (Beckman-Coulter) set to 532 nm. The microplate was incubated for 2-3 hours at 45-50°C and then the optical density of each well was read with the same wavelength. Pre-reading was subtracted from the final reading and data were analysed using a four parameter logistic algorithm to derive concentrations of samples from known standards using SoftMax Pro (Molecular Devices, Sunnyvale, CA, USA).

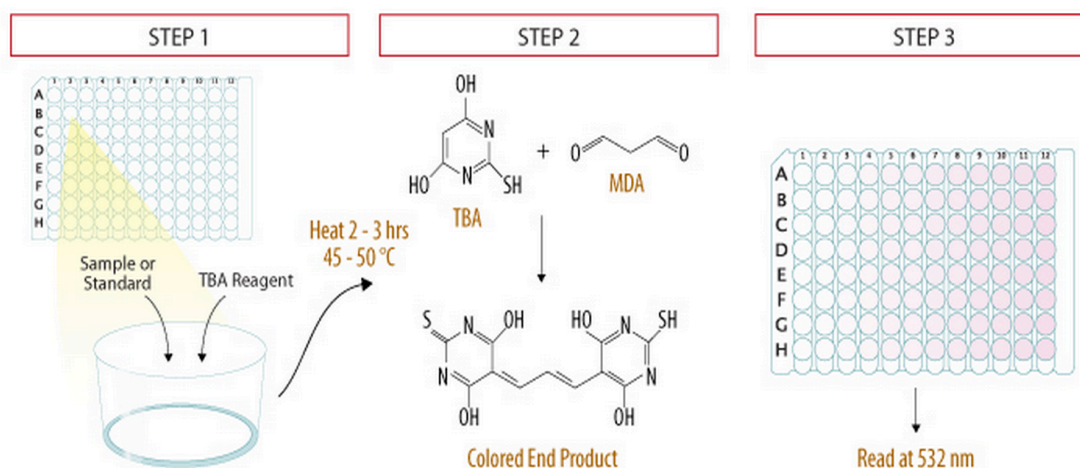


Figure 17. Assay Principle for the TBARS Parameter Kit. **Step1:** Acid-treated samples and standards and the TBA reagent are added to a 96-well microplate. **Step 2:** During 3 hours incubation in the presence of heat (45-50 °C) MDA in the sample reacts with the TBA reagent and produces a coloured end product **Step 3:** The microplate is read at 532nm and the intensity of the colour corresponds to the level of lipid peroxidation in the sample. Picture source: <https://www.rndsystems.com>

2.6 Immunocytochemistry

PFA-fixed cells were first incubated in 50µl of blocking solution comprising 5% normal donkey serum (D9663, Sigma) in PBS containing 0.3% Triton X-100 (T8787, Sigma) for 60 minutes at room temperature. Primary antibodies to measure proliferation, differentiation, apoptosis, transcription factor nuclear translocation (**Table 7**) were diluted in appropriate concentrations of blocking solution buffer and 30µl/well were added and left at 4°C overnight. In the negative control for unspecific binding condition 30µl/well of blocking without primary antibody was added. The next morning, cells were washed, incubated in blocking solution for 30 minutes and then incubated with appropriate fluorescently tagged secondary antibodies (Alexa Fluor 488 donkey anti-rabbit, A-21208, 1:1000; Alexa Fluor 555 donkey anti-mouse, A-315070, 1:1000; both from Life Technologies) at 30µl/well for 2 hours at room temperature. After 3 washes, cells were counter-stained with 0.02mg/ml DAPI at 50µl/well for 5 minutes and washed on 3 more occasions. At least three independent experiments were conducted on three independent cultures, and each condition comprised of six technical replicates.

Antibody (Host species/company)	Dilution	Function
Ki67 <i>(Rabbit/ Ab15580, Abcam)</i>	1:500	Ki67 is a nuclear protein present during all active phases of the cell cycle (G1, S, G2 and mitosis), but absent from the resting cells
CC3 <i>(Rabbit/ 9664, Cell Signalling)</i>	1:500	Cleaved Caspase-3 is a critical executioner of apoptosis
Dcx <i>(Rabbit/ Ab18723, Abcam)</i>	1:1000	Doublecortin is a microtubule-associated protein expressed by neuronal precursor cells and immature neurons
MAP2 <i>(Mouse/ Ab11267, Abcam)</i>	1:500	Microtubule-associated protein 2 is involved in microtubule assemble. This is a neuron-specific cytoskeletal protein that is enriched in dendrites
NF-κB p65 <i>(Mouse/ sc-8008, Santa Cruz)</i>	1:100	NF-κB is a transcription factor involved in regulation of immune-responses and various physiological processes in the cells.

Table 7. List of primary antibodies

2.6.1 Automated quantification of immunofluorescence

An automated approach was employed to quantify cell number, markers of proliferation, differentiation, apoptosis and nuclear translocation of a transcription factor (**Table 7**) using the CellInsight* NXT High Content Screening (HCS) Platform (ThermoScientific). First, images were acquired by autofocus on nuclear staining (Channel 1, DAPI positive, fluorescence in the wavelength 386) in order to determine the field of interest. Next, exposures were adjusted in channels 2 (as revealed by fluorescence at 488nm wavelength) and 3 (as revealed by fluorescence at 555nm wavelength) in order to obtain the optimum exposure times for all wells and conditions across the 96-well plate. Subsequently, images at x10 magnification were acquired for further analysis (**Figure 18**). 15 fields of view per each well were used to quantify images.

For identification of the markers in the nucleus (Ki67, NF- κ B p65) only labelling confined within the nuclear perimeter as stipulated by DAPI staining was quantified (**Figure 19a** and **20**). For quantification of the markers expressed in the cell body and dendrites (CC3, Dcx and MAP2) the software relies upon nuclear staining to aid construction of a concentric circle around the immediate vicinity of the nucleus. The creation of such a circle creates a map or region of interest. Staining confined within the region between the outer circle (edge of cell body) and inner circle (outside of the nucleus) was deemed positive (**Figure 19b** and **20**).

Based on values from the negative staining controls and good examples of positive staining, distinct and robust thresholds were set for average intensity of target regions of interest (e.g. nuclear or cell body) to delineate positive populations in both channel two and three. Any cell with an average intensity bigger than the threshold set was deemed positive. Crucially, the same settings were applied to each well upon

scanning of the plate, significantly reducing counting bias. Moreover, these same settings were applied to all corresponding plates within an experiment, ensuring reproducibility and further unbiased comparisons.

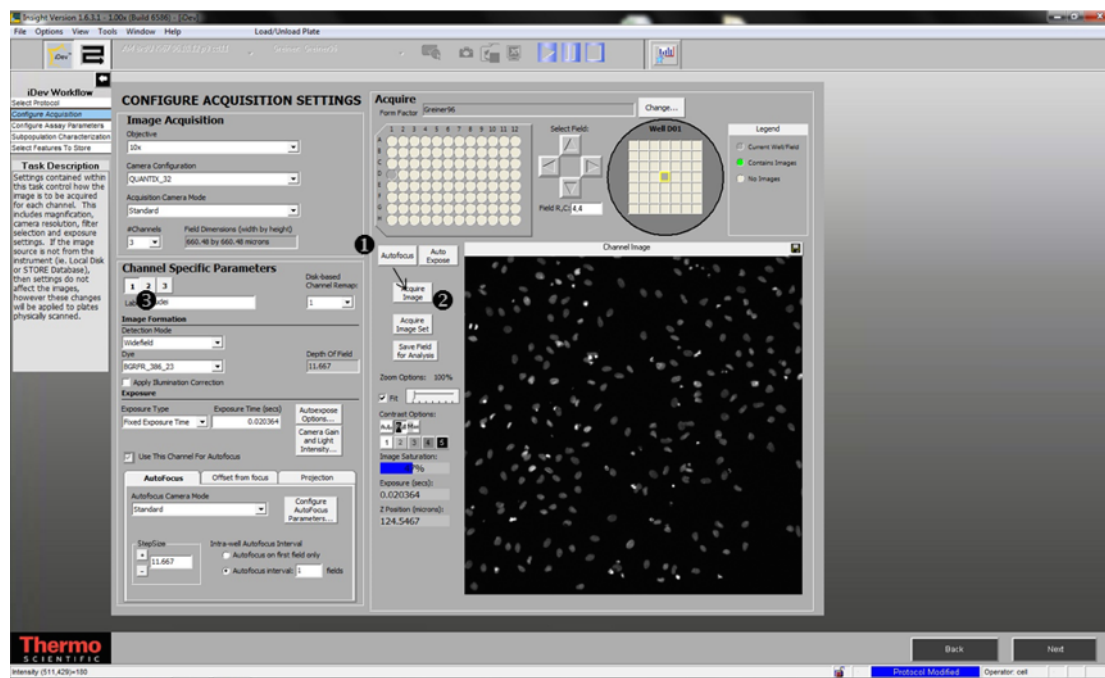
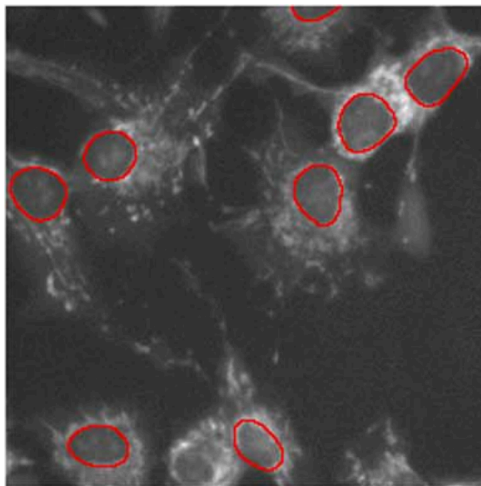
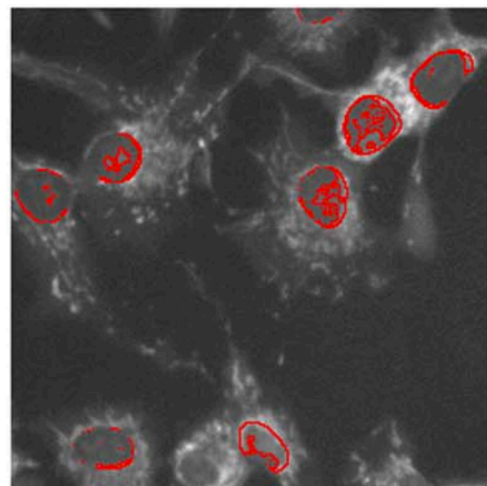


Figure 18. An automated approach to quantifying the number of cells and markers of interest using the Cell Insight Platform (ThermoScientific). Images are acquired by autofocus on nuclear staining Channel 1 (wavelength 386nm) and then exposures are adjusted in channels 2 (wavelength 488nm) and 3 (wavelength 555nm) followed by obtaining images at x10 magnification for further analysis.

a)

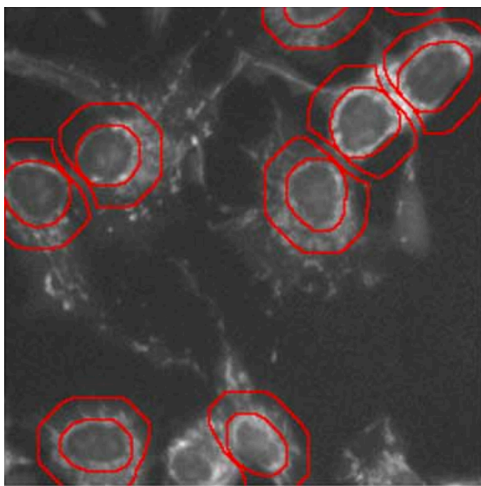


circle around the nucleus

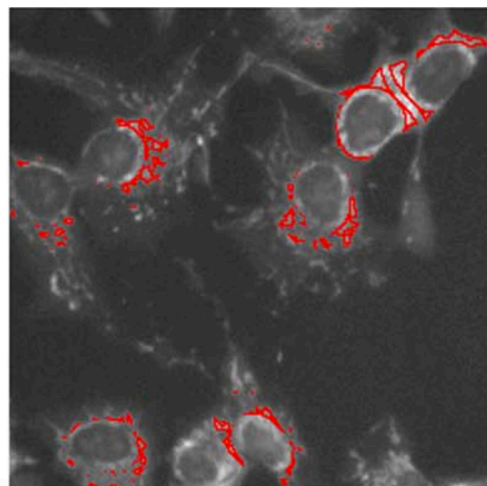


staining that is deemed positive within the nucleus

b)



circle around and outside of the nucleus (both manually adjustable by the user)



staining that is deemed positive between the nucleus and the circle and outside of the nucleus

Figure 19. Example of an automated identification of the area of interest. a) For identification of the markers in the nucleus, circle around the nucleus is formed and labelling confined within the circle is quantified. **b)** For quantification of the markers expressed in the cell body and dendrites, circle around the nucleus and around the immediate vicinity of the nucleus is formed. Staining within the area between the outer circle (edge of cell body) and inner circle (outside of the nucleus) is quantified.

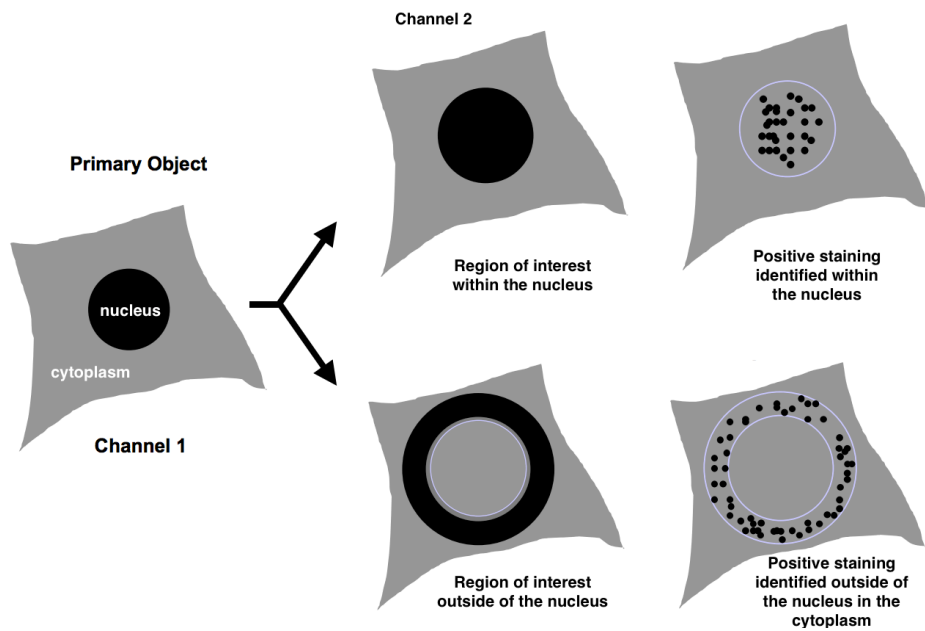


Figure 20. Algorithm of an automated identification of the area of interest. First, the software identifies the primary object - the nucleus of the cell, stained with DAPI. Then according to the user's assigned region of interest – either within the nucleus, or outside of the nucleus, the software quantifies positive staining.

2.6.2 Representative images

Figure 21 displays representative images of immunolabelling of the human hippocampal progenitor cells (HPC0A07/03C) as captured with the Cell Insight Imaging Platform (ThermoScientific): a) neuroblasts (Dcx-positive) and immature neurons (MAP2-positive); b) proliferating cells (Ki67-positive); c) nuclear translocation of the NF- κ B p65 subunit; d) apoptotic Cleaved Caspase 3 (CC3-positive).

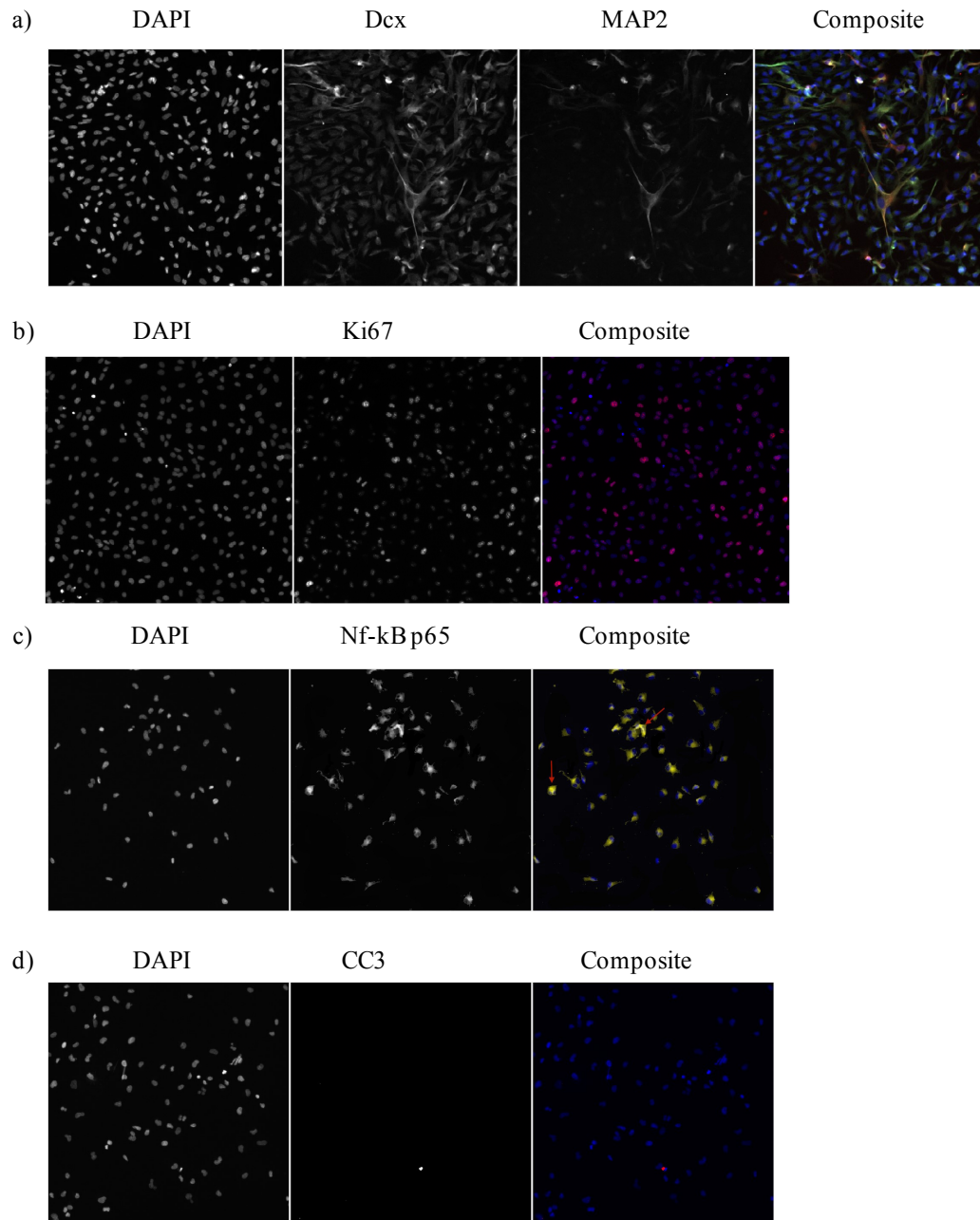


Figure 21. Representative images of neurogenic markers acquired using the Cell Insight Imaging Platform (ThermoScientific): **a)** immature neurons detected by Dcx (green) and MAP2 (red) **b)** staining proliferating cells as labelled Ki67 (purple) **c)** apoptotic cell death revealed by the CC3 (red) and **d)** nuclear translocation of NF- κ B p65 subunit (yellow). 15 fields of view per each well were used to quantify images.

2.7 Secreted protein quantification using ELISA method

Cells were plated in 96 well plates (Nunc) at a density of 1.5×10^4 cells per well in 100 μ L of RMM and allowed to firmly attach overnight (**Table 6**). Then cells were treated with 1 μ M, 10 μ M, 25 μ M, 50 μ M, 100 μ M, 200 μ M 500 μ M and 1 mM of *t*-BHP (200 μ L per well) for 1 hour and supernatant was collected. Cells were then washed and fresh media was added for another 23 hours and supernatant was collected again. Upon supernatant collection samples were immediately transferred to storage in aliquots to avoid future multiple freeze/thaw cycles at -80°C for later measurement. At least three independent experiments were conducted on three independent cultures with four technical replicates per condition, and each sample was tested in duplicate. IL-6 secreted into the supernatant (both after 1 hour and after subsequent 23 hours) by the cells treated with *t*-BHP was quantified using the human IL-6 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Abingdon, UK). The procedure was performed according to the manufacturer's instructions. Briefly, cell supernatant and standard dilutions were incubated in wells pre-coated with IL-6 antibody for two hours. IL-6 conjugate, a polyclonal antibody specific for human IL-6 conjugated to horseradish peroxidase, was then added and incubated for two hours. Finally, a substrate solution of TMB was incubated for thirty minutes causing a colour change, before being terminated with a stop solution of sulphuric acid. Absorbance was read at 450 nm using a DTX 880 Multimode Detector (Beckman-Coulter). Data were analysed using a four parameter logistic algorithm to derive concentrations of samples from known standards using SoftMax Pro (Molecular Devices, Sunnyvale, CA, USA).

2.8 Multiplex protein quantification using Luminex platform

Experiments were conducted and samples were stored as outlined in section 2.7. Cells supernatants were run on the Human Cytokine Magnetic 25-Plex Panel (Invitrogen) according to the manufacturer's instructions. Briefly, samples were incubated with 25µL of a suspension of antibody beads containing 25 antibodies to different chemokines and cytokines for two hours, along with standard curves, generated by serial dilution (containing all 25 immunoproteins assayed). Antibodies form primary complexes in proportion to the amount of soluble immunoprotein specific to that antibody. Each antibody is covalently bound to magnetic microspheres which are dyed with red and infrared fluorospheres (known as a bead region), which allows for differentiation of one bead (and therefore the bound antibody) from another using infrared lasers. Samples were then incubated with a biotinylated detector antibody (100µL per well) for one hour. The final incubation step consists of 100µL of Streptavidin-RPE solution in each well. The samples were then read on a Luminex 200 platform which using the principle of flow cytometry to read individual beads on two characteristics: their bead region (determined by infrared lasers) and their fluorescent intensity (proportional to the amount of original immunoprotein (**Figure 22**)). Data was analysed with a four-parameter logistic algorithm to derive concentrations of the samples from known standards using SoftMax Pro (Molecular Devices).

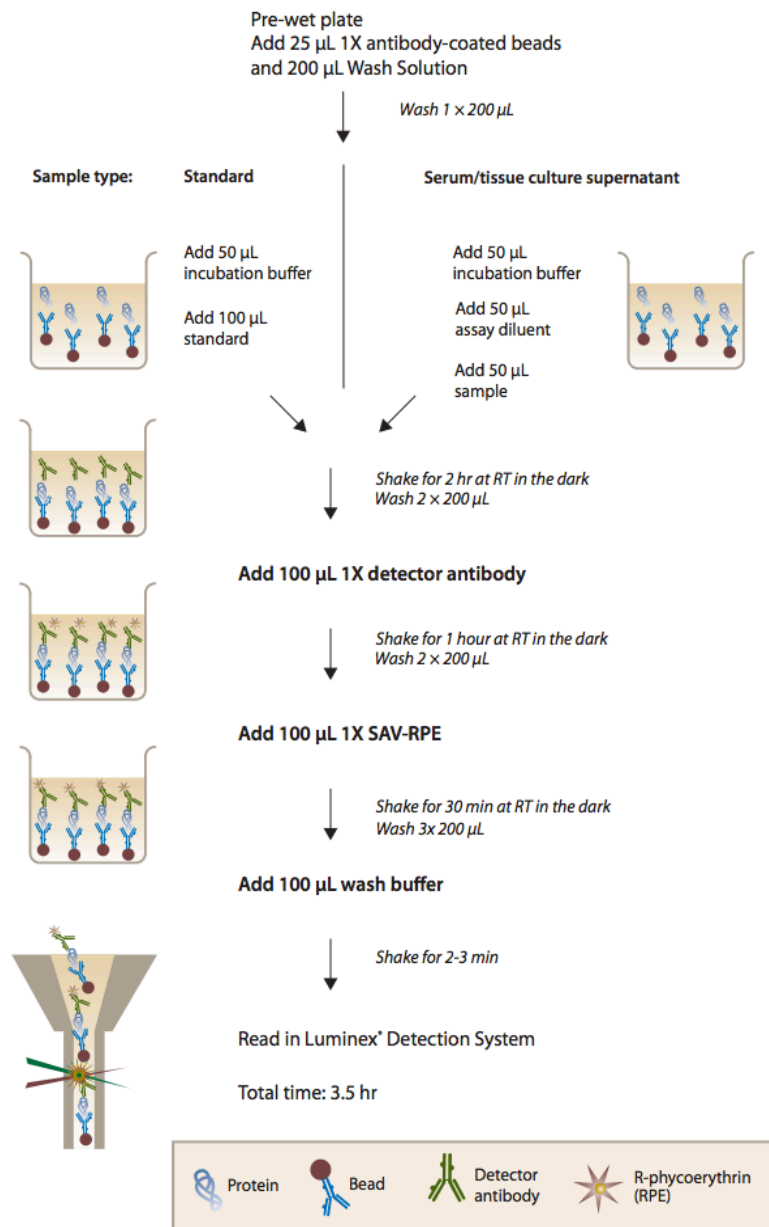


Figure 22. Cytokine Human Magnetic 25-Plex Panel protocol. Samples and standards were incubated in turn with antibody-bound beads to 25 immunoproteins, followed by incubation with a biotinylated detector antibody and finally with a streptavidin-RPE solution that produces a fluorescent signal in proportion to the amount of original immunoprotein. This signal is then read, along with an infrared marker distinguishing immunoprotein antibodies from each other, by the Luminex xMAP system working on the principle of flow cytometry. Picture source: <https://tools.thermofisher.com>

2.9 Nrf2 transactivation assay

2.9.1 Nuclear protein extraction

Nuclear extracts were obtained using a Nuclear Extraction kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. All manipulations were performed on ice. First, cells were washed once in 5ml ice cold PBS. Cells were then scraped in 5ml ice-cold PBS, transferred to 15ml centrifuge tubes and centrifuged at 3000 rpm for 10 min in a pre-cooled centrifuge at 4°C. The supernatant was carefully discarded and each cell pellet was gently resuspended in 400µl hypotonic buffer. Samples were transferred to 1.5ml microcentrifuge tubes and incubated for 15 min on ice. 20µl of detergent was added and samples were vortexed for 10 seconds at the highest setting. The suspensions were then centrifuged at 14,000 x g for 2 min at 4°C and the supernatant (cytoplasmic fraction) was removed. Cell pellets (nuclear pellets) were resuspended in 50µl of complete lysis buffer containing protease inhibitors (Active Motif) and vortexed for 30 seconds at the highest setting. The samples were incubated for 30 min at 4°C on a rocking platform at 400 rpm. Afterwards, the lysates were vortexed for 1 min at the highest setting and centrifuged for 10 min at 14,000 x g in a pre-cooled microcentrifuge at 4°C. The supernatants (nuclear fraction) were transferred to a fresh microcentrifuge tube and stored at -80°C until further analysis.

2.9.2 Protein quantification

Protein concentrations were quantified using a bicinchoninic acid (BCA) colorimetric assay system (Novagen). Protein samples were diluted 1:10 in protein lysis buffer and

incubated with the kit reaction mixture in a ratio of 1:8 (sample: reaction mixture) for 30 min at 37°C. Absorbance was measured with a microplate reader (DTX 880 Multimode Detector, Beckman Coulter, Brea, USA) at 562nm and the protein concentration of each sample in duplicate was determined by comparison with a bovine serum albumin (BSA) standard curve (0µg/ml, 1.25µg/ml, 5µg/ml, 10µg/ml, 25µg/ml, 75µg/ml, 125 µg/ml, 250µg/ml, 500µg/ml, 750µg/ml) using SoftMax Pro (Molecular Devices).

2.9.3 TransAM Nrf2 assay

Nuclear extracts were obtained after treatment of the cells with 25µM 50µM and 100µM of *t*-BHP for 1 hour in 75cm² flasks (**Table 6**). Samples were thawed on ice and diluted to a final concentration of 5µg per condition directly before the assay performance. Nrf2 binding to the ARE consensus binding site (5'-GTCACAGTGA CT CAG-CAGAATCTG -3') was analysed using the TransAM Nrf2 assay (Active Motif). The active form of Nrf2 contained in nuclear extract (obtained as described in **2.9.1**) specifically binds to this oligonucleotide. The primary antibody used to detect Nrf2 recognises an epitope on Nrf2 protein upon DNA binding. Addition of an HRP-conjugated secondary antibody provides a colorimetric readout quantified by spectrophotometry. 40µL of complete binding buffer was added to each well and 5µg of nuclear protein extracts or provided positive control extracts diluted in complete lysis buffer or plain complete lysis buffer for blank wells were added to the wells accordingly. Nuclear protein extracts were incubated with the Nrf2 oligonucleotide in binding buffer for 1 hour at room temperature on a rocking platform set for 100 rpm. Plates were washed three times with wash buffer and

sequentially incubated with 100 μ L of Nrf2 antibody (1:1000) for 1 hour at room temperature without agitation. Plates were washed again three times with wash buffer and 100 μ L of HRP-conjugated antibody (1:1000) was added and incubated for 1 hour at room temperature without agitation. Plates were washed now four times with wash buffer and 100 μ L of warmed to room temperature developing solution was added. After protected from light 15 minutes incubation at room temperature 100 μ L of stop solution was added. Then the absorbance was read with a spectrophotometer (DTX 880 Multimode Detector, Beckman Coulter, Brea, USA) at 450 nm with a reference wavelength 655nm (**Figure 23**). Specificity of the assay was confirmed using wild-type and mutated oligonucleotide sequences.

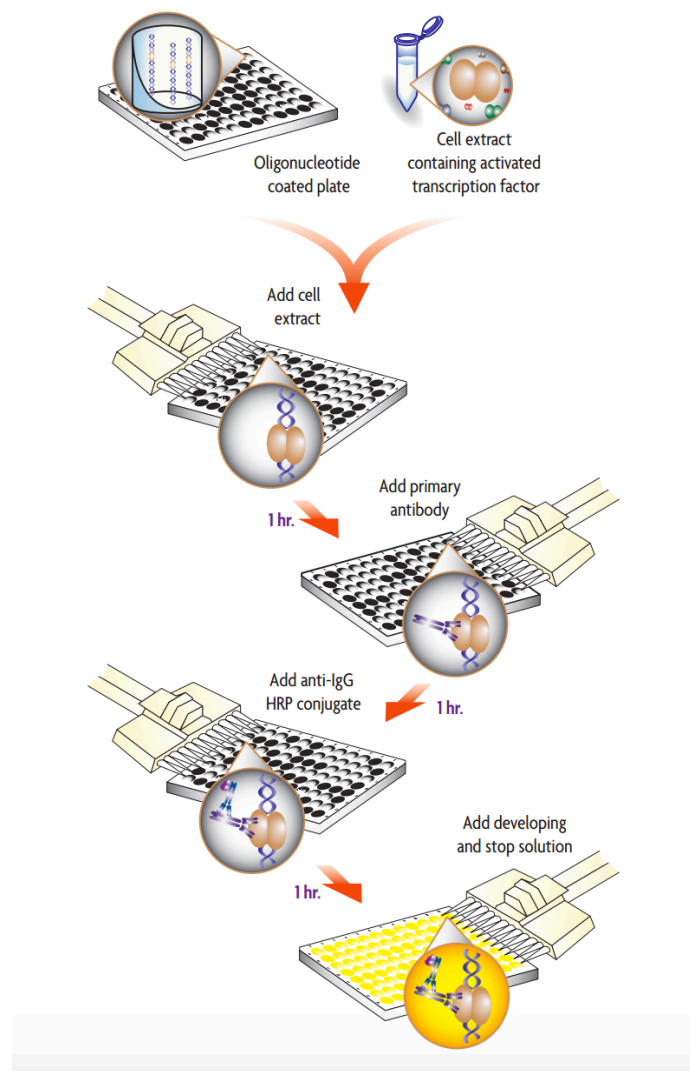


Figure 23. Flow chart of Nrf2 transactivation assay using the Nrf2 TransAM kit from Active Motif. Nuclear protein extracts were incubated with the Nrf2 response element oligonucleotide on the assay plate and sequentially incubated with Nrf2-antibody and with HRP-conjugated antibody for 1 hour at room temperature. Absorbance was measured to quantify the amount of Nrf2 bound to the Nrf2 sequence on the plate. Picture source: www.activemotif.com

2.10 Electrochemiluminescence assay using Meso Scale Discovery platform

2.10.1 Whole cell lysate extraction

Experiments were conducted in 6-well plates as outlined in section 2.3.4 (**Figure 16b, Table 6**). Whole cell lysates were obtained according to the manufacture's recommendations and all manipulations were performed on ice. Briefly, cells were washed once with ice cold PBS, then 1ml of PBS was added in the wells and cells were scraped from the surface of the dish and transferred into 15ml conical tubes. Cells were centrifuged at 500 x g for 3 minutes at 4°C, the supernatant was removed and the pellet was resuspended in 500µL of complete lysis buffer, containing protease and phosphatase inhibitors (**Table 8**). Cell lysate was immediately transferred to storage at -80°C for later measurement. Directly before the assay performance protein concentrations were quantified using a bicinchoninic acid (BCA) colorimetric assay system (Novagen) as described in **2.9.2**.

Buffer	Reagents
Tris Lysis Buffer (1X Incomplete)	150mM NaCl 20mM Tris, pH 7.5 1mM EDTA 1mM EGTA 1% Triton-X-100
Complete Lysis Buffer	10ml 1X Tris Lysis Buffer 100μL Phosphatase Inhibitor I 100μL Phosphatase Inhibitor II 100μL Protease Inhibitor Solution

Table 8. Complete Lysis Buffer.

2.10.2 Meso Scale Discovery assay

To compare the levels of phosphorylated MAP kinases across treatment conditions and vehicle the MAP Kinase Phosphoprotein Assay Whole Cell Lysate Kit (MSD[®] MULTI-SPOT Assay System) was used. This is a multiplex sandwich immunoassay, where an array of capture antibodies against different targets – phospho-p38, phospho-ERK1/2, phospho-JNK is patterned on distinct spots in the same well (**Figure 24**). Analytes in the samples bind to these capture antibodies and detection antibodies against the targets that are added over the course of incubation periods and are conjugated with an electrochemiluminescence compound - MSD SULFO-TAG label, bind to the sample analyte. Upon addition of the MSD read buffer appropriate chemical environment for the electrochemiluminescence is provided. Then the plate is loaded into an MSD SECTOR[®] Imager for analysis. Electrochemiluminescent labels generate light when voltage is applied to the plate electrodes and the instrument measures intensity of the emitted light to provide a quantitative measure of phospho-p38, phospho-ERK1/2, phospho-JNK present in the sample (**Figure 25**).

Cell lysate samples were thawed on ice and diluted to a final concentration of 20µg per well. The assay procedure was performed according to the manufacturer's instructions. Briefly, 150µl of blocking solution was added into each well and the plate was incubated for 1 hour with vigorous shaking at 1000 rpm at room temperature. The plate was washed three times with tris wash buffer, then 25µL of samples was added and the plate was incubated for three hours with vigorous shaking at 1000 rpm at room temperature. The plate was washed again three times with tris wash buffer and 25µL of detection antibody solution was added and the plate was incubated for 1 hour with vigorous shaking at 1000 rpm at room temperature. The

plate was washed again three times with tris wash buffer, then 150 μ L of 1xread buffer was added to each well and the plate was analysed in the SECTOR Imager.

2.11 Gene expression analysis

2.11.1 RNA isolation

Experiments were conducted in 6-well plates as outlined in section 2.3.2, (**Figure 15c, Table 6**). For analysis of gene expression changes, RNA was isolated using RNeasy micro kit Qiagen, Crawley, UK) according to the manufacturer's instructions. HPC0A07/03C cells were grown in a monolayer in 6-well plates flasks (Nunc) until ~80% confluence and the cells were lysed in 350µl lysis buffer RLT (Qiagen) with addition of 3.5µl of β-mercaptoethanol and physically removed from the plates. One volume (350µl) of 70% molecular biology grade ethanol (Sigma) was added to the lysate and the solution was mixed well by pipetting. Each sample (700µl) was transferred to an RNeasy spin column. The spin column was placed into a 2ml collection tube and centrifuged for 1min at 10,000 rpm. The flow through was discarded and 700µl of wash buffer RW1 was added to the RNeasy spin column. The spin column was centrifuged for 1 min at 10,000 rpm and 500µl buffer RPE was added and centrifuged once for 1 min at 10,000 rpm. Another 500µl of buffer RPE was added and spin columns were centrifuged for 2min at 10,000 rpm. The spin columns were placed into a clean 2 ml collection tube and centrifuged for 1min at 13,000 rpm to eliminate any possible carryover of buffer RPE, or any residual flow-through remains on the outside of the spin column which may impair RNA quality. To elute the RNA from the spin column, spin columns were then placed into 1.5ml collection tubes and 30µl of RNase-free water was added. Columns were centrifuged for 1min at 10,000 rpm and the RNA-containing eluate was stored at - 80°C until further processing.

The concentration and purity of extracted nucleic acids was measured using the NanoDrop™ 1000 spectrophotometer (Thermo Scientific) directly before performing reverse transcription. The 260/280 and 260/230 absorbance ratios were used to assess the purity of RNA. A 260/280 ratio ≈ 2.0 and a 260/230 ratio in range of 1.8-2.2 indicate pure RNA; ≈ 2.0 for 260/280 ratio and ≈ 1.8 for 260/230 ratio values were accepted.

2.11.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Complimentary DNA (cDNA) was synthesised using SuperScript® III Reverse Transcriptase (18080-044, Life Technologies). 1 µg of RNA was combined with 250 ng of random hexamers (N8080127, Life Technologies) and 1mM dNTP mix (R0191, Thermo Scientific) made up to 13µl with nuclease-free H₂O (Sigma-Aldrich). The mix was incubated for 5 minutes at 65°C on a heated block to denature RNA secondary structure. It was then promptly placed on ice and incubated for 1 minute. The mix was then made up to 20µl with the following reagents: 1x First Strand Buffer (Invitrogen), 5mM dithiothreitol (18080-044, Life Technologies), 40 units RNaseOUT™ (10777, Life Technologies), 200 units SuperScript® III Reverse Transcriptase (Invitrogen) and 3µl of nuclease-free H₂O (Sigma-Aldrich). Samples were incubated at 25°C for 5 minutes, 50°C for 1 hour (optimal temperature of Superscript® III), 55°C for 30 minutes (to remove any secondary structures) and finally 70 °C for 15 minutes to terminate the reaction. For qPCR applications, samples were diluted to a concentration of 1:10 in nuclease-free H₂O.

2.11.3 Quantitative Real-Time PCR (qPCR)

Quantitative Real-Time PCR (qPCR) was performed using commercially available TaqMan[®] assays (Thermo Fisher Scientific). Each assay includes target primers and a sequence specific probe optimized for the best functional performance of the assay, therefore it is a sensitive and specific method for gene expression analysis. The company does not divulge primer sequences. 10uL of qPCR reaction mix per one assay consisted of the reagents and cDNA template presented in the **Table 9**.

qPCR was run on Chromo4[™] Real-Time PCR detector (Bio-Rad) using the following parameters:

Initial denaturation: 50°C for 2 minutes

Denaturation: 95°C for 10 minutes

Annealing: 95°C for 15 seconds

Extension: 60°C for 1 minute

Annealing and Extension cycles were repeated 40 times. Fluorescence was recorded at the end of each cycle.

Reagent	Volume
20xTaqMan [®] Gene Expression Assay	0.5µL
TaqMan [®] Gene Expression Master Mix	5µL
cDNA template sample	4µL
RNase-free water	0.5µL

Table 9. qPCR reaction mix

First, to choose the reference genes appropriate for my specific experimental conditions five housekeeping genes previously used with HPC0A07/03C cell line in our laboratory (beta-actin (*ACTB*), *RPL13A*, *GAPDH*, *RPLP2* and *YWHAZ*) were tested with control and each experimental condition in three independent experiments. *ACTB* and *RPL13A* were chosen as the reference genes for subsequent experiments due to the smallest variability in its expression across all conditions and replicates (cycle threshold variability was within 0.5).

Each target gene (*NQO1*, *SLC1A1*, *HMOX1*, *SLC7A11*, *GCLM*, *Keap1*) and two reference genes (*ACTB* and *RPL13A*) assays were validated by creating a standard curve, consisting of five serial dilutions. Each sample was assayed in triplicate and each target was normalized to the geometric mean of the two reference genes. The Pfaffl Method (Pfaffl, 2001) was used to determine relative target gene expression. Data are expressed as fold change from the vehicle treated control condition.

2.12 Kynurenine pathway quantification

2.12.1 Sample preparation

Experiments were conducted as outlined in **2.7 (Table 6)**. Both supernatants after 1 hour and after subsequent 23 hours were measured. Perchloric acid 0.5M (HClO_4), was used to extract analytes from cell culture medium. 1ml of cell supernatant from each experimental condition was centrifuged ($2000 \times \text{rpm}$, 5 min) to pellet and remove dead cells. 100 μL of each supernatant sample was collected and combined with an equal volume of ice cold solvent (1M HClO_4) fortified with internal standard (final concentration 1 μM); then samples were centrifuged ($15000 \times \text{g}$, 10 min) and the

supernatants were collected and stored at -80 °C until high performance liquid chromatography (HPLC) analysis. The supernatants were directly injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) device.

2.12.2 Liquid chromatography

The analysis was performed using an Agilent HP 1200 liquid chromatograph (Agilent, Milan) consisting of a binary pump, an autosampler and a thermostated column compartment. Chromatographic separations were carried out using a Discovery HS-F5 column (150 x 4.6 mm, 5 µm) using 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) as mobile phase. The HPLC analyses were carried out using a linear elution profile of 15 minutes from 5% to 90% of acetonitrile (ACN). The column was washed with 90 % of acetonitrile for 3.5 min followed by the equilibration of the column for 5 min with 5 % acetonitrile. The flow rate was 0.5 ml/min. The injection volume was 40µL. An Agilent 6410 triple quadrupole-mass spectrometer with an electrospray ion source operated in positive mode was used for detection. Flow injection analysis was used to optimise the fragmentor and source parameters. The optimized source parameters for MS analysis were: drying gas temperature 350 °C and gas flow 12 L/min, nebulizer gas flow pressure 35 psi and capillary voltage 4500 V.

2.13 Statistical analysis

For cell viability assays, at least 3 independent experiments performed on different

experimental days (biological replicates, indicated as 'n') with 4 technical replicates (four different wells on the same plate in the same experiment) were conducted for each set of experiments. For lipid peroxidation assay 4 independent experiments conducted on different experimental days were performed and TBARS assay was conducted in duplicates for each sample. For proliferation and differentiation immunocytochemistry assays at least 3 independent experiments performed on different experimental days with 6 technical replicates were conducted for each set of experiments. For measurement of cytokine levels in the supernatant by ELISA or Luminex multiplex immunoassay at least 3 independent experiments performed on different experimental days with 4 technical replicates were conducted for each set of experiments and the assay was performed in duplicates. For Nrf2 transactivation assay at least 4 independent experiments performed on different experimental days were conducted and the assay was performed in duplicates. For electrochemiluminescence assay at least 3 independent experiments performed on different experimental days were conducted and the assay was performed in duplicates. For gene expression analysis, at least 5 independent experiments performed on different experimental days were conducted. One RT-PCR was conducted per sample per experiment, and quantitative Real-Time PCR was conducted in triplicates for each sample.

Data in each individual experiment was normalized to the average value of the vehicle condition. The percentage change from the vehicle condition is presented as the mean value \pm SEM. All statistical analyses were performed with GraphPad Prism 6.0g (Graph Pad Inc., La Jolla, CA, USA) on independent biological replicates (indicated as 'n'). One-Way ANOVA with Newman-Keuls post hoc test was used for multiple comparisons between treatment groups and controls. Student's t-test was used to

compare means of two independent treatment groups. P-values <0.05 were considered significant.

2.14 Drugs and reagents

All drugs and reagents were purchased from Sigma–Aldrich (St Louis, MO, USA), unless otherwise stated. Growth factors EGF and bFGF were purchased from Peprotech (London, UK). FR180204 ERK1/2 inhibitor was purchased from Selleckchem (USA) and dissolved in DMSO. Sertraline hydrochloride, venlafaxine hydrochloride, EPA (>98% pure, extracted and purified from fish), DHA (>98% pure, extracted and purified from algal vegetable oil), were dissolved in 100% ethanol (EtOH). Ketamine hydrochloride, *N*-acetyl-L-cysteine and glutathione reduced ethyl ester were dissolved in RMM.

3 Results

3.1 Oxidative stress induced by *t*-BHP causes cell damage in human progenitor hippocampal cells

3.1.1 *t*-BHP causes cell damage and cell death in a dose-dependent manner, with intensity of effects dependant on time of exposure

In order to establish the experimental set up of induced OS in the human progenitor hippocampal cell line, I first sought to identify the doses of *t*-BHP that cause significant cell damage. As described in current literature, toxic doses of *t*-BHP vary with both the density of cells and the cell types studied (Chen and Chen, 2011; Steinbrenner et al., 2006). Therefore, initially cells were treated in 96-well plates with a range of concentrations of *t*-BHP from 1µM to 1mM for 24 hours and then cell viability was assessed using the MTS assay. No significant change in cell viability was observed at 1µM, 2.5µM, 5µM, 7.5µM and 10µM concentrations of *t*-BHP. A significant reduction in cell viability to 65%, $p < 0.05$ occurred at 25µM of *t*-BHP, and this reduction increased reaching 30%, $p < 0.0001$ at 50µM of *t*-BHP. At 75µM, 100µM and 1mM doses of *t*-BHP the reduction in cell viability reached only 5% and less ($p < 0.0001$) suggesting that almost all cells died because of the massive oxidative damage inflicted by the peroxide (**Figure 26**).

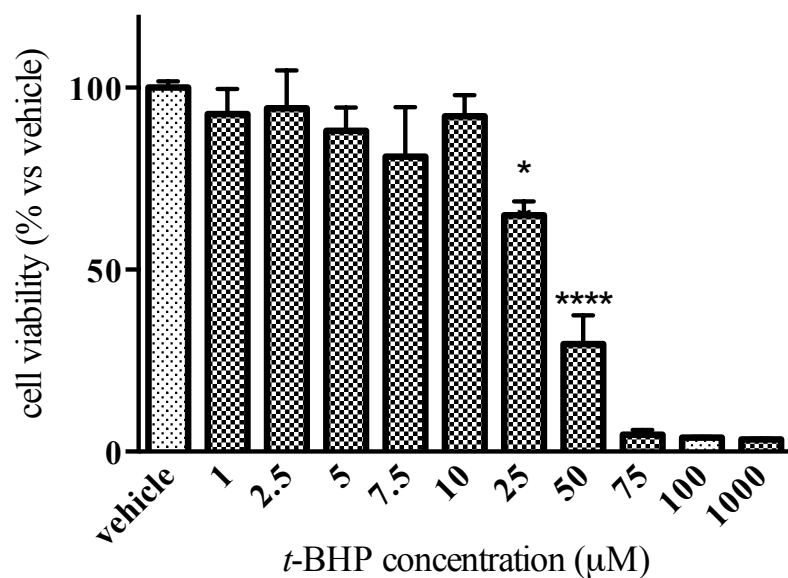


Figure 26. Dose-dependent decrease in cell viability after 24 hours of *t*-BHP treatment. A significant decrease in cell viability was observed at 25μM to 65% and at 50μM to 30%, while at 75μM, 100μM and 1000μM of *t*-BHP almost all cells died. Data are shown as mean ± SEM, * $p < 0.05$, **** $p < 0.0001$, compared with vehicle treatment. Three independent experiments were conducted on three independent cultures (n=3).

Given such drastic decreases in cell viability, and hoping for more controlled reductions, I changed the experimental design, modifying the time of exposure. In this new paradigm I treated cells with the same range of concentrations of *t*-BHP for 1 hour, then cells were cultured in fresh media for the following 23 hours, keeping the same total culture time before the MTS assay was performed (so 24 hours in total). Evaluating which experimental set up is more physiologically relevant is not straightforward; in my first one I was identifying cell damage straight after the OS insult, in the second one I determined if cells survived after a certain period subsequent to the inflicted OS insult. This last acute 1 hour treatment with *t*-BHP caused a significant delayed decrease in cell viability in a dose-dependent manner when compared with vehicle: at 100 μ M to 71%, at 200 μ M to 42%, at 500 μ M to 28% ($p<0.0001$) and at 1mM to 14% ($p<0.0001$) (**Figure 27**).

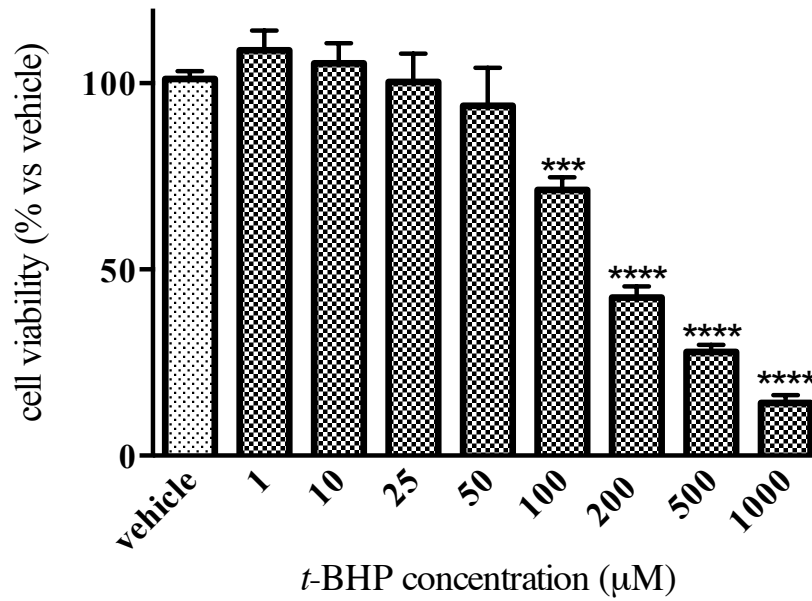


Figure 27. Dose-dependent decrease of cell viability after 1 hour of *t*-BHP treatment followed by 23 hours of culturing in fresh media. A significant decrease in cell viability was observed starting at 100μM of *t*-BHP: at 100μM to 71%, at 200μM to 42%, at 500μM to 28% ($p < 0.0001$) and at 1mM to 14%. Data are shown as mean \pm SEM, *** $p < 0.001$, **** $p < 0.0001$, compared with vehicle treatment. Four independent experiments were conducted on four independent cultures ($n=4$).

Given these reductions in cell viability after treatment with *t*-BHP for 1 hour followed by 23 hours with no treatment (fresh media), two doses of *t*-BHP, 100µM and 500µM, were selected to be used in those subsequent experiments designed to identify compounds that can prevent damage. Since there was no significant decrease in viability when cells were treated using this paradigm with 1µM, 10µM, 25µM or 50µM of *t*-BHP, these concentrations were used in subsequent experiments to study cell signalling pathways as I hypothesized that mild redox shifts in the system towards more a oxidised environment would cause upregulation of cellular antioxidative defences and influence the cell fate.

3.1.2 *t*-BHP induces lipid peroxidation in a dose-dependent manner

In order to check whether *t*-BHP causes lipid peroxidation in the human hippocampal progenitor cells I measured thiobarbituric acid reactive substances, performing the TBARS assay. Cells were treated in 6-well plates with 50µM, 100µM, 200µM, 500µM and 1000µM of *t*-BHP for 1 hour, then the supernatant was removed and stored at -80°C until analysis. Results are presented as percentage change of concentration of TBARS from the vehicle. A significant increase in the level of TBARS was observed in cells treated with 500µM (+56%, $p < 0.001$) and 1000µM of *t*-BHP (+96%, $p < 0.0001$). Although at 50µM, 100µM and 200µM of *t*-BHP statistical significance was not reached, a clear trend was observed (**Figure 28**).

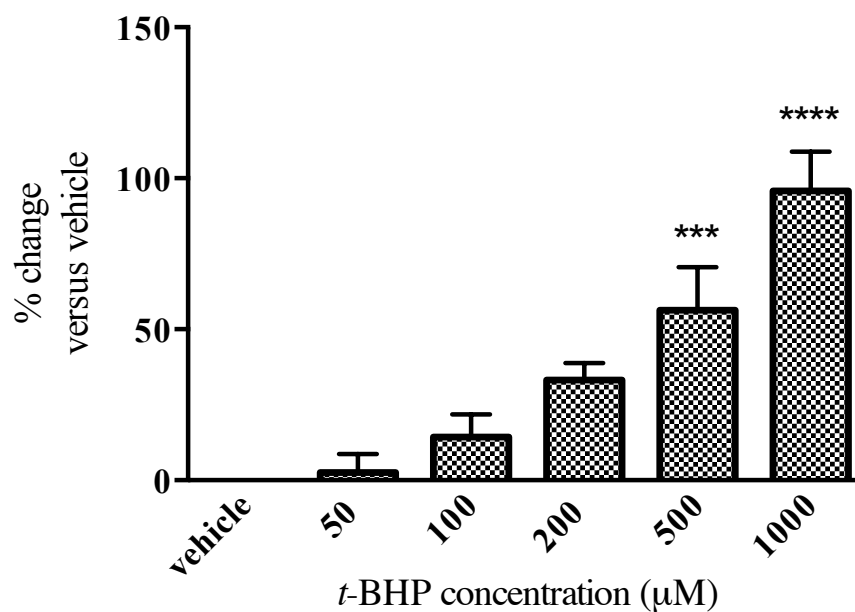


Figure 28. Dose-dependent increase in lipid peroxidation after 1 hour *t*-BHP treatment. A significant increase in lipid peroxidation measured through thiobarbituric acid reactive substances was observed at 500μM and at 1000μM. Data are shown as mean ± SEM, ***p<0.001, ****p<0.0001, compared with vehicle treatment. Four independent experiments were conducted on four independent cultures (n=4).

3.1.3 *t*-BHP treatment does not cause differences in expression of Cleaved Caspase-3

To investigate if the damage induced by *t*-BHP on the human hippocampal progenitor cells involved apoptotic mechanisms, cells were treated in 96-well plates with 10µM, 25µM, 50µM, and 100µM of *t*-BHP for 1 hour. Cells were then washed and fresh media was added for another 23 hours. After 24 hours in total, cells were fixed with 4% PFA in preparation for immunocytochemistry. To assess apoptosis, cells were immunolabelled with Cleaved Caspase-3 (CC3), a commonly used apoptotic marker, and staining was analysed using the automated approach CellInsight* NXT HCS Platform. While treatment with *t*-BHP caused a significant reduction in the number of cells at all concentrations evaluated (**Figure 29a**), there were no significant differences in the expression levels of CC3 (**Figure 29b**). Results are expressed as percentage of CC3 positive cells over total number of cells in each condition.

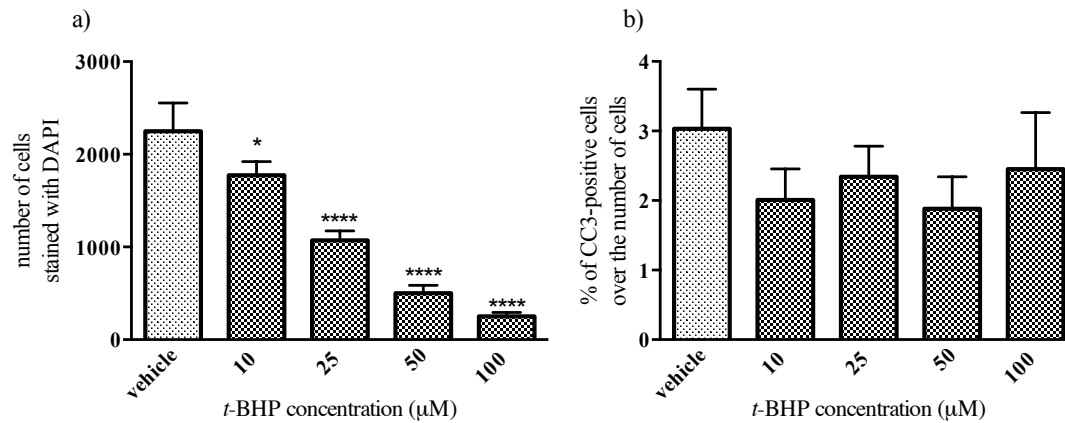


Figure 29. Treatment of cells with *t*-BHP for 1 hour followed by 23 hours of culturing in fresh media did not cause differences in the expression levels of Cleaved Caspase-3. a) The total number of cells was significantly lower upon treatment with *t*-BHP at doses of 10 μ M and higher. **b)** No significant differences in the level of expression of CC3 were observed across conditions when compared to vehicle. Data are shown as mean \pm SEM, * p <0.05, **** p <0.0001, compared with vehicle treatment. Three independent experiments were conducted on three independent cultures (n=3).

3.2 Effects of *t*-BHP on neurogenesis

My second aim was to explore how neurogenesis is affected in the human hippocampal progenitor cells upon induction of redox shifts by *t*-BHP treatment and to identify potential signalling pathways involved. Increased OS biomarkers in depressed patients have been reported across many different studies (Black et al., 2015; Liu et al., 2015) and there is also evidence of impaired neurogenesis in MDD coming mainly from animal models (Lucassen et al., 2016). However, it is not clear how alterations in redox homeostasis might influence neurogenesis, in particular in the human hippocampal progenitor cells. Current literature suggests that adult neurogenesis transiently generates OS (Walton et al., 2012) and neural stem cells (NSC) are extremely sensitive to redox regulation and maintain a high ROS status for implementation of the normal cellular functioning (Le Belle et al., 2011). Thus, I sought to induce redox shifts in the human hippocampal progenitor cells by *t*-BHP treatment and assess the levels of proliferation and differentiation in the neural progenitors by labelling them with relevant markers.

3.2.1 Effects of *t*-BHP treatment on proliferation

In order to investigate the effects of *t*-BHP on proliferation, I first treated proliferating human hippocampal progenitor cells for 72 hours with *t*-BHP at concentrations of 1 μ M, 10 μ M, 25 μ M, 50 μ M and 100 μ M. After this incubation time cells were fixed with 4% PFA in preparation for immunocytochemistry. Cells were immunolabelled with Ki67, a marker of cellular proliferation, and images were obtained and analysed using the CellInsight* approach. Treatment with *t*-BHP showed no distinct effects on cell proliferation at 1 μ M and 10 μ M, causing a decrease in proliferation at 25 μ M

(72%, $p < 0.01$) and at 50 μ M (37%, $p < 0.0001$) (**Figure 30a**). Data is presented as the percentage of Ki67-positive cells in treated cultures compared to those in control cultures. At 100 μ M almost all cells died (**Figure 30b**).

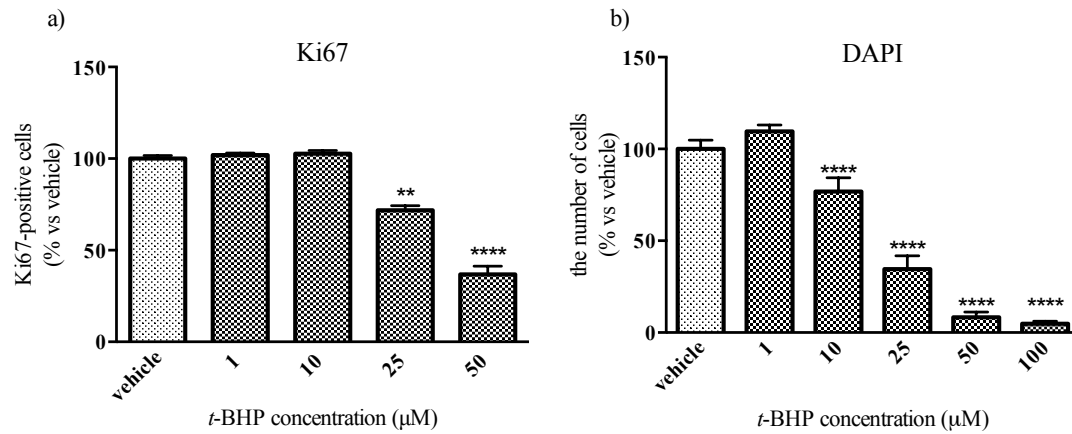


Figure 30. Treatment of human hippocampal progenitor cells with *t*-BHP for 72 hours of proliferation caused decrease in Ki67-positive cells. a) A significant decrease in Ki67-positive cells was observed at 25μM (72%) and at 50μM (37%). **b)** A significant decrease in the total number of cells was observed at 10μM (77%), at 25μM (35%), at 50μM (8%) and at 100μM (5%). Data are shown as mean ± SEM, **p<0.01, ****p<0.0001, compared with vehicle treatment. Three independent experiments were conducted on three independent cultures (n=3).

3.2.2 Effects of *t*-BHP treatment on differentiation

To examine the effects of *t*-BHP on neuronal differentiation and neuronal maturation, I treated the human hippocampal progenitor cells in 96-well plates with 1 μ M, 10 μ M, 25 μ M, 50 μ M, 100 μ M of *t*-BHP for 72 hours during proliferation (in the presence of growth factors) and for subsequent 7 days during differentiation (in the absence of growth factors) as described in 2.3.4. Thus, cells were treated continuously during both the proliferation and the differentiation phase for 10 days in total. After fixation of the cells upon completion of the incubation time, immunocytochemistry was conducted to visualize young Dcx-positive neuroblasts and mature MAP2-positive neurons and also analysed using the CellInsight* NXT HCS Platform. Treatment with *t*-BHP led to a significant increase in the amount of Dcx-positive neuroblasts when treated with 1 μ M (+38%, $p < 0.01$) and 10 μ M (+30%, $p < 0.05$). No effect was observed at 25 μ M of *t*-BHP (**Figure 31a**). The percentage of MAP2-positive neurons rose when treated with 1 μ M (+34%, $p < 0.05$) and 10 μ M (+33%, $p < 0.05$), whereas at 25 μ M of *t*-BHP no significant difference was observed (**Figure 31b**). Treatment with *t*-BHP during these 10 days caused a significant decrease in the total number of cells when exposed to a dose of 25 μ M (50%, $p < 0.0001$), with higher decreases observed at 50 μ M and 100 μ M of *t*-BHP, where almost all cells died (**Figure 31c**). Data is presented as percentage change from the vehicle, calculated as the number of Dcx-positive and MAP2-positive cells in each condition over the number of cells (stained with DAPI) in the corresponding wells (**Figure 31a, b**). For the number of cells data is presented as the percentage in treated cultures compared to those in control cultures (**Figure 31c**).

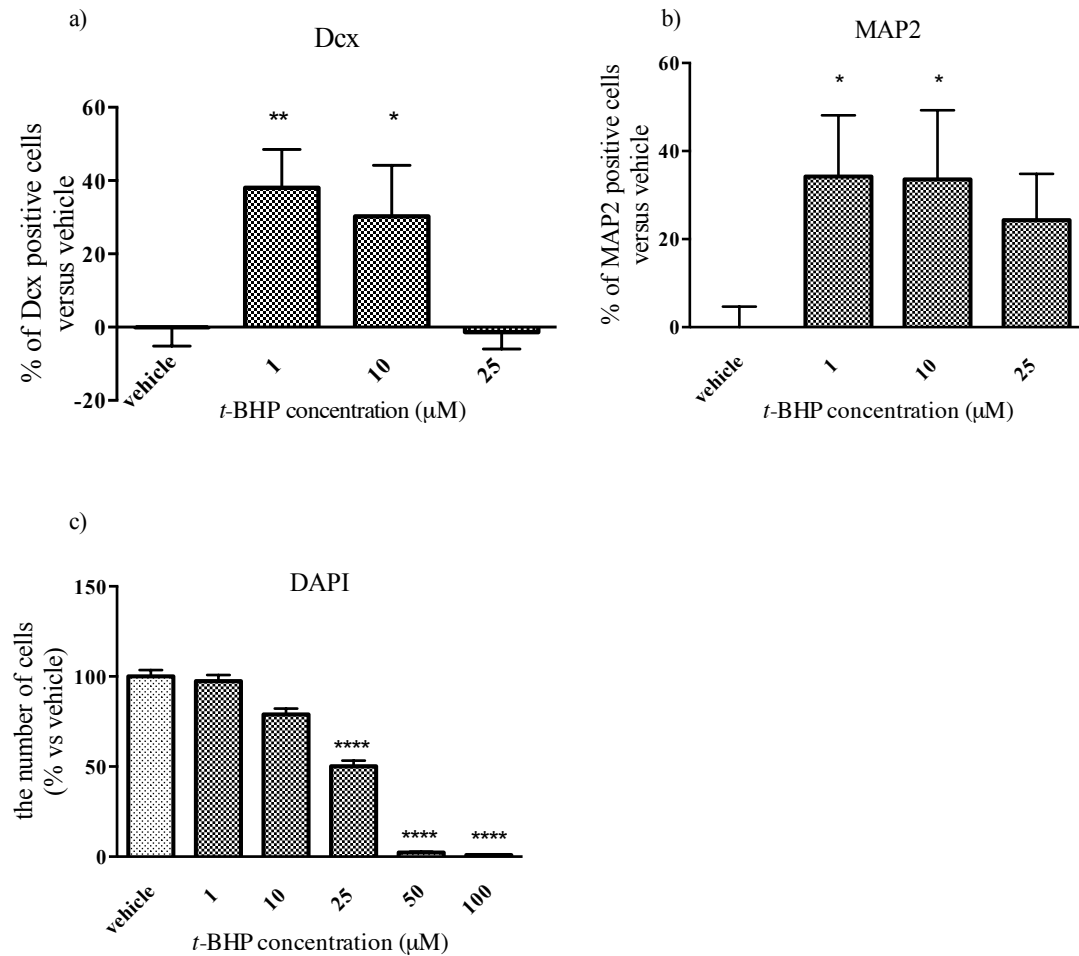


Figure 31. Treatment of human hippocampal progenitor cells with *t*-BHP during proliferation and differentiation caused increase in neurogenesis and decrease in the number of cells. a) A significant increase in the amount of Dcx-positive cells was observed at 1μM (+38%) and at 10μM (+30%); **b)** A significant increase in the amount of MAP2-positive cells was observed at 1μM (+34%) and at 10μM (+33%); **c)** A significant decrease in the number of cells was observed at 25μM to 50%, and almost all cells died at 50μM and at 100μM. Data are shown as mean ± SEM, * $p < 0.05$ ** $p < 0.01$, **** $p < 0.0001$, compared with vehicle treatment. Six independent experiments were conducted on six independent cultures (n=6).

3.3 Modulating effect of *t*-BHP treatment on MAP kinases

I wished to investigate whether the effects of *t*-BHP on progenitor cell differentiation are mediated through MAP kinase signalling pathways. Therefore, I first sought to explore whether treatment of the cells with *t*-BHP would lead to an increase in phosphorylation, indicative of activation of p38, ERK1/2 and JNK kinases. My intention was to then block activation (if any observed) with relevant inhibitors and examine whether it would abrogate the pro-neurogenic effects induced by *t*-BHP treatment. To compare the levels of these phosphorylated MAP kinases across treatment conditions and vehicle I treated cells in 6-well plates in order to obtain enough protein (**Table 6**) with the same concentrations of *t*-BHP and in exactly the same manner as in **3.2.2 (Figure 16b)**. Upon 10 days of total incubation time, whole cell lysates were obtained as described in methods **2.10.1.** and stored at -80°C until the assay performance. All cell lysates were thawed at once directly before the assay and protein concentration was calculated with the BSA method to then normalise across conditions for the electrochemiluminescence immunoassay assay, using the MAP Kinase Phosphoprotein Assay Whole Cell Lysate Kit (MSD[®] MULTI-SPOT Assay System). Treatment with *t*-BHP did not lead to a significant increase in phospho-p38, phospho-ERK1/2 or phospho-JNK kinases in the human progenitor hippocampal cells. However, a trend was observed in phospho-ERK1/2 (**Figure 32**).

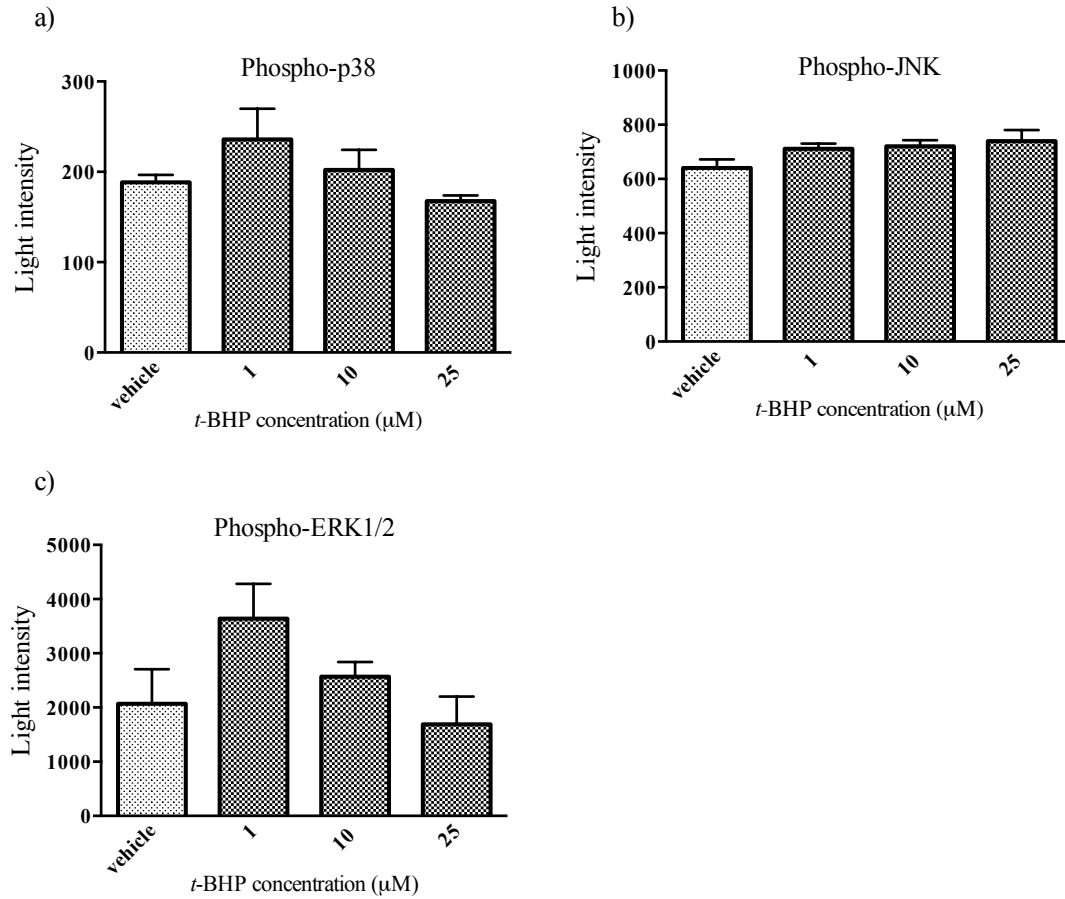


Figure 32. *t*-BHP treatment during proliferation and differentiation did not cause changes in the levels of phosphorylated MAP kinases. Treatment with 1 μ M, 10 μ M and 25 μ M of *t*-BHP did not lead to significant changes in the levels of expression of phospho-p38, phospho-ERK1/2 and phospho-JNK kinases in the human hippocampal progenitor cells. Data are shown as mean \pm SEM. Three independent experiments were conducted on three independent cultures (n=3).

To examine whether the effect of *t*-BHP on MAP kinase activation could be time-dependant, I repeated the experiment treating cells with *t*-BHP for 24 hours only, using the two lowest concentrations of the peroxide this time, due to the high cost of the assay. Upon 24 hours of total incubation the same procedures were repeated as described above. Again no significant increase in expression of the kinases under investigation was observed, only showing a similar trend in the level of the phospho-ERK1/2 (**Figure 33**).

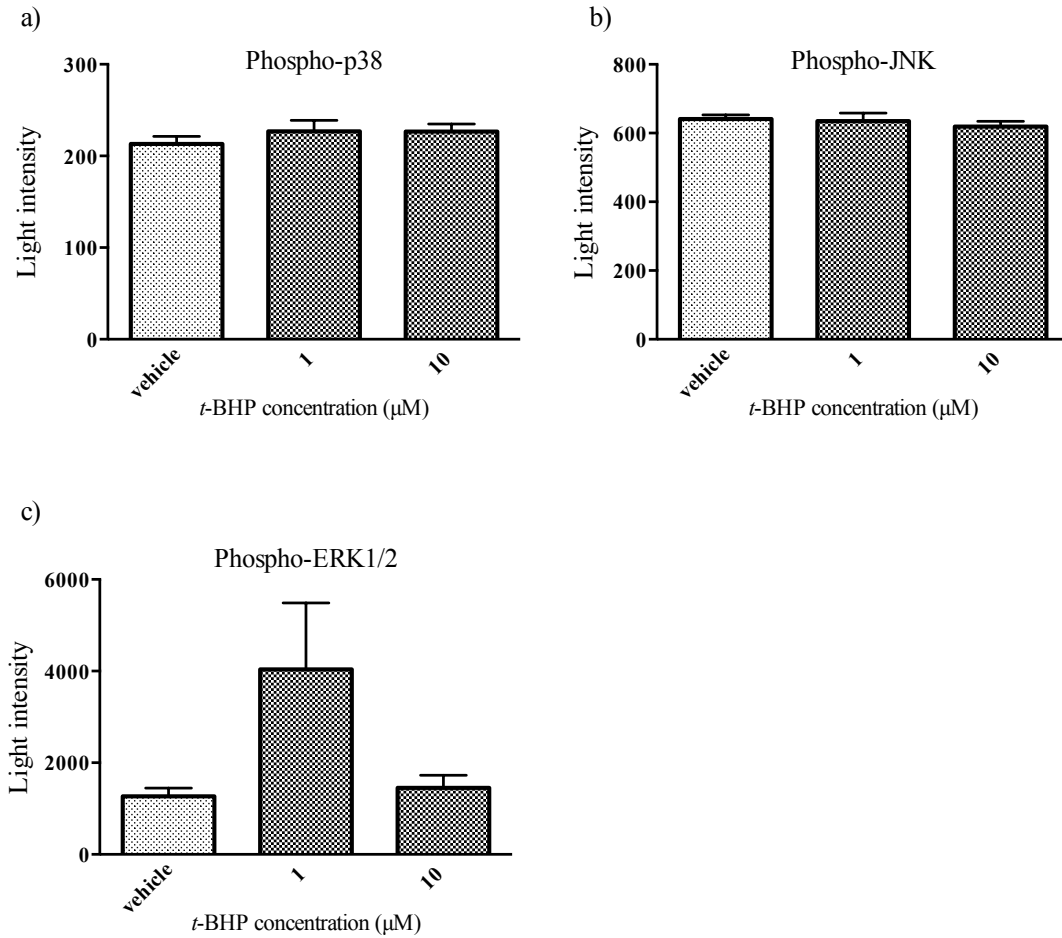


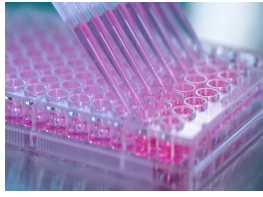
Figure 33. *t*-BHP treatment for 24 hours during proliferation did not cause changes in the levels of phosphorylated MAP kinases. Treatment with 1μM and 10μM of *t*-BHP did not lead to significant changes in the levels of expression of phospho-p38, phospho-ERK1/2 and phospho-JNK kinases in the human hippocampal progenitor cells. Data are shown as mean \pm SEM. Three independent experiments were conducted on three independent cultures (n=3).

3.3.1 Inhibition of ERK1/2 abrogated the increase in MAP2-positive cells induced by *t*-BHP

Although the increase in phospho-ERK1/2 did not reach statistical significance, based on the signal obtained and due to the expenses of the assay I decided to proceed and block the phospho-ERK1/2 with the FR180204 inhibitor and investigate whether it would prevent the *t*-BHP-induced changes in neurogenesis. Thus, there was a slight modification to the experimental design described in 2.3.4. Cells were first pre-treated in 96-well plates with 10nM or 100nM of FR180204 for 24 hours and then co-treated with either 1μM or 10μM of *t*-BHP for the following 48 hours during proliferation. After 72 hours of proliferation cells were washed and differentiated with the same conditions as in proliferation, so co-treated with 10nM or 100nM of FR180204 with either 1μM or 10μM of *t*-BHP. To test if FR180204 inhibitor had an effect on its own, cells were also treated with FR180204 only. Treatment with 1μM and 10μM of *t*-BHP increased the amount of MAP2-positive cells (+21%, $p<0.01$, and +27%, $p<0.0001$, respectively) and the amount of Dcx-positive cells (+32%, $p<0.001$ and +33%, $p<0.01$, respectively) when treated with 1μM and 10μM of *t*-BHP. Treatment with the inhibitor alone showed no effect.

I then evaluated the effect of pre-treatment with the inhibitor. Cells were treated with 10nM and 100nM of FR180204 inhibitor for 24 hours, followed by co-treatment with 1μM or 10μM of *t*-BHP during 48 hours of proliferation and 7 days of differentiation (**Figure 34**). This pre-treatment with the inhibitor did not lead to significant changes in the amount of Dcx-positive cells when compared to the cells treated with *t*-BHP only (**Figure 35**). When cells were pre-treated with 10nM of FR180204, a trend towards a decrease in the amount of MAP2 positive cells was observed, reaching significance with the higher concentration of the inhibitor. Pre-treatment with 100nM

of FR180204 and co-treatment with 10 μ M of *t*-BHP caused a complete reduction in the amount of MAP2 positive cells ($p<0.01$), when compared to the condition where cells were treated with 10 μ M of *t*-BHP only. Also, a clear trend towards a decrease in the amount of MAP2 positive cells was observed when cells were pre-treated with 100nM of the FR180204 and co-treated with 1 μ M of *t*-BHP, however a significant difference was not reached. Data is presented as percentage change from the vehicle (**Figure 36**).



Cells are seeded and incubated overnight in a 96-well plate

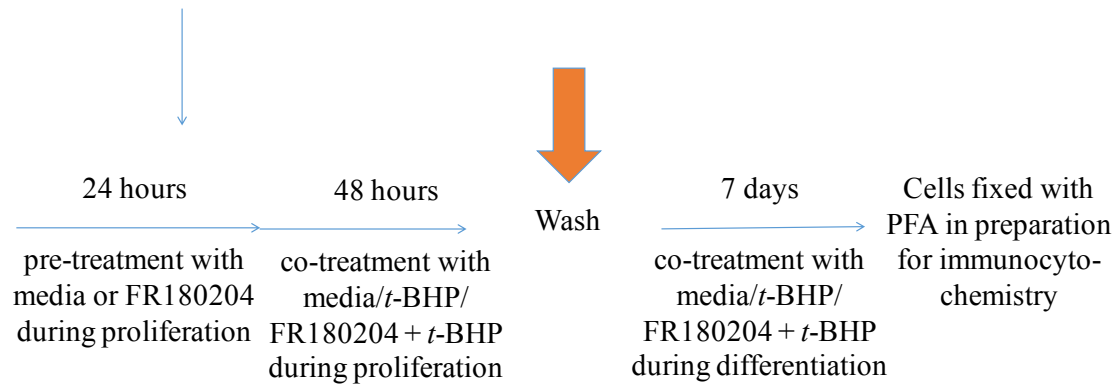


Figure 34. Experimental design of the assay with FR180204 – ERK1/2 inhibitor.

Human hippocampal progenitor cells were seeded in 96-well plates and incubated overnight. Cells were then either pre-treated with media or FR180204 inhibitor for 24 hours and subsequently co-treated with media, *t*-BHP or *t*-BHP plus FR180204 inhibitor for the next 48 hours. After 72 hours of proliferation cells were washed with warm DMEM and the same treatment conditions in media without growth factors were applied for the next 7 days. After 10 days of total incubation time cells were fixed with PFA in preparation for immunocytochemistry.

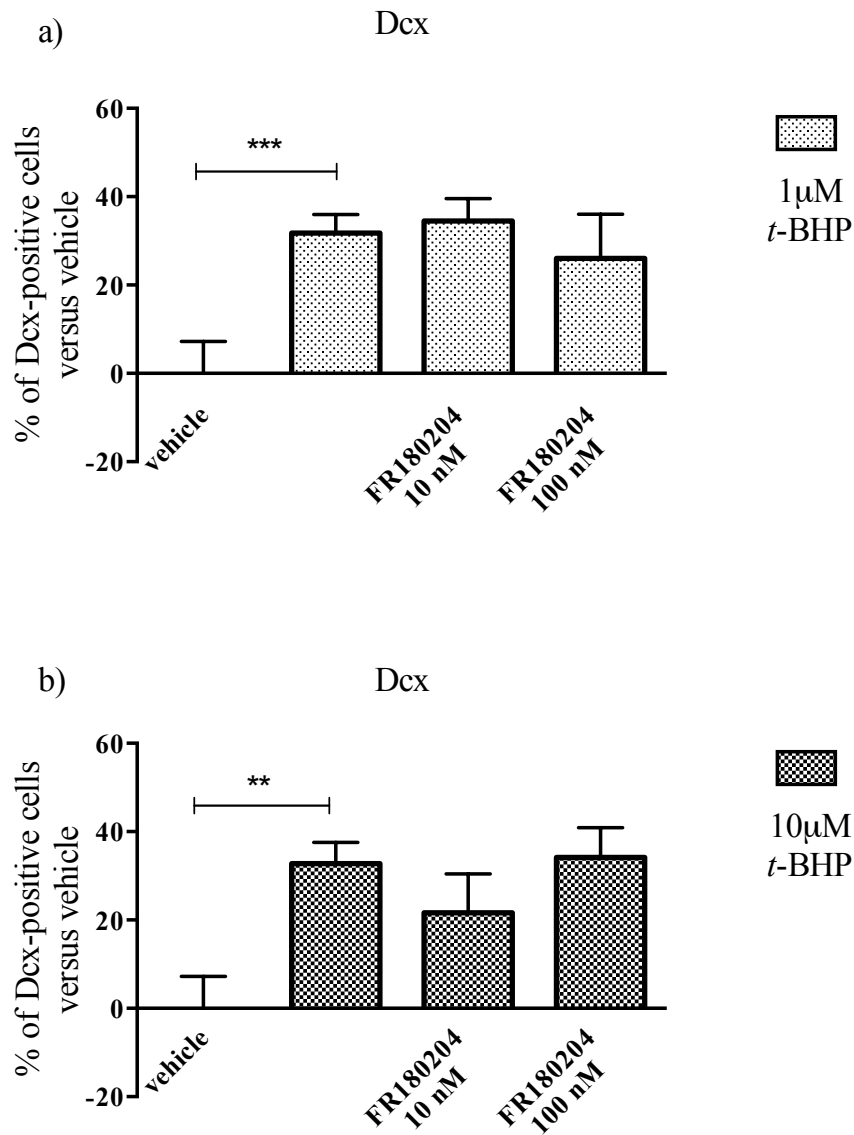


Figure 35. *t*-BHP-induced increase of neuroblasts and the effect of FR180204 inhibitor. A significant increase in the amount of Dcx-positive cells was observed at **a)** 1 μ M (+32%) and at **b)** 10 μ M (+33%). **a, b)** FR180204 inhibitor did not affect *t*-BHP-induced increase in the amount of Dcx-positive cells. Data are shown as mean \pm SEM, ** $p < 0.01$. Three independent experiments were conducted on three independent cultures (n=3).

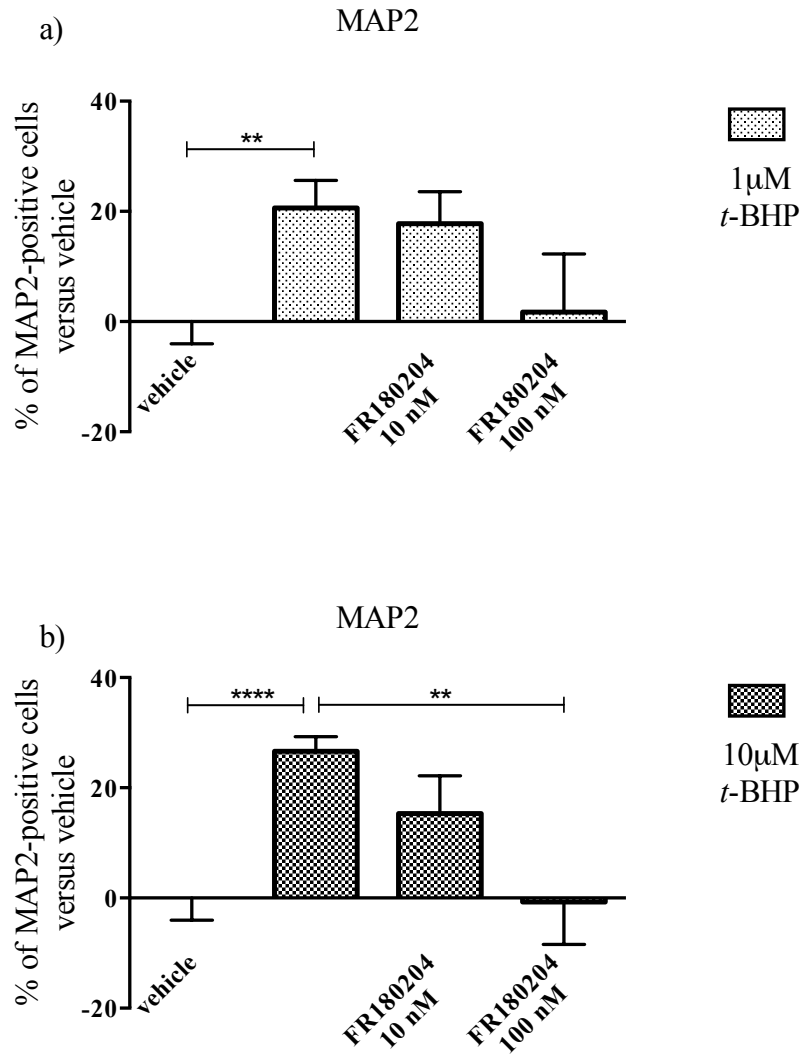


Figure 36. Abrogation of *t*-BHP-induced increase of neuronal maturation by blocking ERK1/2 with FR180204 inhibitor. **a)** A significant increase in the amount of MAP2-positive cells was observed at 1 μ M (+21%). **b)** A significant increase in the amount of MAP2-positive cells was observed at 10 μ M (+27%). 100nM of FR180204 in the presence of 10 μ M of *t*-BHP completely prevented an increase in MAP2-positive cells caused by *t*-BHP and was significantly different from the condition treated with 10 μ M of *t*-BHP. Data are shown as mean \pm SEM, ** p <0.01, **** p <0.0001. Three independent experiments were conducted on three independent cultures (n=3).

3.4 Oxidative stress induced by *t*-BHP causes activation of pleiotropic transcription factors NF- κ B and Nrf2

The inducible regulation of gene expression through pleiotropic transcription factors is a central element of the normal cell physiology and is key to the ability of multicellular organisms to adapt to various environmental stresses. NF- κ B and Nrf2 are two redox-sensitive transcription factors critical for a myriad of interrelating events determining the cell fate (**Figure 8**) (Bakunina et al., 2015). In particular, Nrf2 is a major antioxidant transcription factor and its activation leads to up-regulation of its antioxidative downstream genes. NF- κ B is a master regulator of immune responses and it also broadly influences gene expression that impact cell survival, differentiation and proliferation (Hayden and Ghosh, 2008). The activation time of these transcription factors depends on the biological context and I decided to investigate whether NF- κ B is activated already after 1 hour of oxidative stimulus and whether it stays activated within 24 hours in the presence of oxidative stimulus. Based on NF- κ B activation after 1 hour and 24 hours, my plan was to choose the time-points for future experiments to measure cytokine expression, hypothesizing that the more pronounced NF- κ B expression the higher the production of cytokines would be. I tested Nrf2 redox-activation after 1 hour only because I wanted to evaluate whether the effects I had observed for the compounds under investigation in the cell viability assay were associated with alterations in gene expression downstream of Nrf2.

3.4.1 *t*-BHP induces Nrf2 nuclear translocation

To examine if Nrf2 transcription factor is activated in the human progenitor hippocampal cells I performed the transactivation assay the TransAM Nrf2 assay

(Active Motif). As described in methods (section **2.9.3**), cells were treated in 75cm² flasks with 25µM, 50µM and 100µM of *t*-BHP for 1 hour and then nuclear extracts were obtained and stored at -80°C until further analysis. After normalisation of the protein concentration across samples the transactivation assay was performed. A significant increase in the level of nuclear Nrf2 was observed in cells treated with 50µM and 100µM of *t*-BHP (+46%, p<0.01 and +113% p<0.0001 respectively) when compared to vehicle (**Figure 37**).

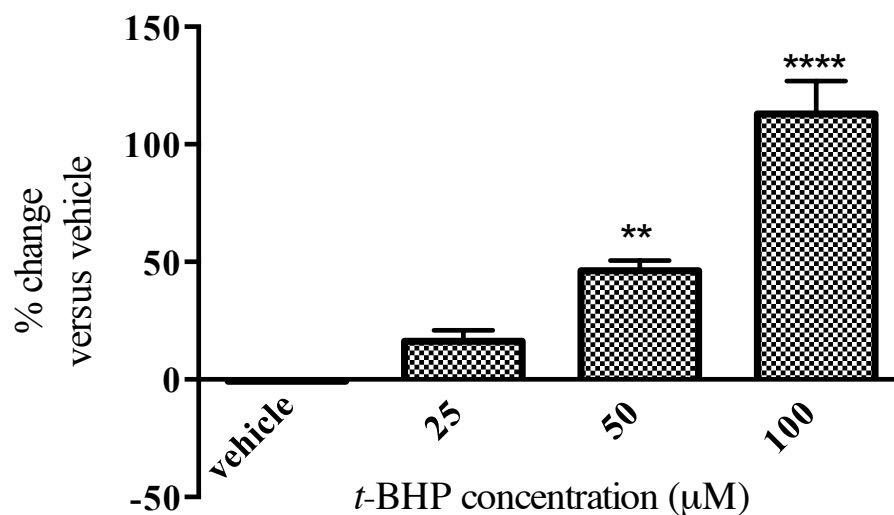


Figure 37. Dose-dependent increase of the amount of Nrf2 in nuclear protein extracts after 1 hour of *t*-BHP treatment. A significant increase in the amount of Nrf2 was observed at 50μM (+46%) and at 100μM (+113%). Data are shown as mean \pm SEM, ** $p < 0.01$, **** $p < 0.0001$, compared with vehicle treatment. Four independent experiments were conducted on four independent cultures (n=4).

3.4.2 *t*-BHP induces NF- κ B p65 subunit nuclear translocation

In order to investigate if treatment of the human hippocampal progenitor cells with *t*-BHP leads to activation of the NF- κ B transcription factor, I followed the nuclear translocation of its p65 subunit by immunostaining. Upon activation the p65 subunit is released from the cytoplasm, translocates to the nucleus and binds to its nuclear localization sequence, initiating transcription of several downstream genes. In this experiment cells were treated for 1 hour with *t*-BHP at concentrations of 25 μ M, 50 μ M and 100 μ M of *t*-BHP and then cells were fixed with PFA in preparation for immunocytochemistry. To investigate whether redox NF- κ B activation occurs at lower concentrations of *t*-BHP and stays for a longer period of time, in a separate experiment cells were treated with 1 μ M and 10 μ M of *t*-BHP for 24 hours. Considering that higher concentrations of *t*-BHP are toxic when applied for 24 hours (**Figure 26**), they were not tested in this experiment.

The images were obtained and analysed using the CellInsight* automated approach. Treatment of the cells with *t*-BHP for 1 hour caused significant increase in the NF- κ B p65 subunit positive cells within the nucleus at 50 μ M and 100 μ M reaching 16% and 17% and being significantly different when compared to vehicle ($p < 0.0001$) (**Figure 38a**). Treatment of the cells with *t*-BHP for 24 hours caused a much smaller, but significant increase in the NF- κ B p65 subunit positive cells within the nucleus at 10 μ M to 4% ($p < 0.05$) with no effect at 1 μ M when compared to vehicle (**Figure 38b**). Data is presented as percentage of NF- κ B p65 positive cells, considered as those with staining detected in the nucleus only, over the total number of cells (stained with DAPI) in the corresponding wells, and analysed versus vehicle.

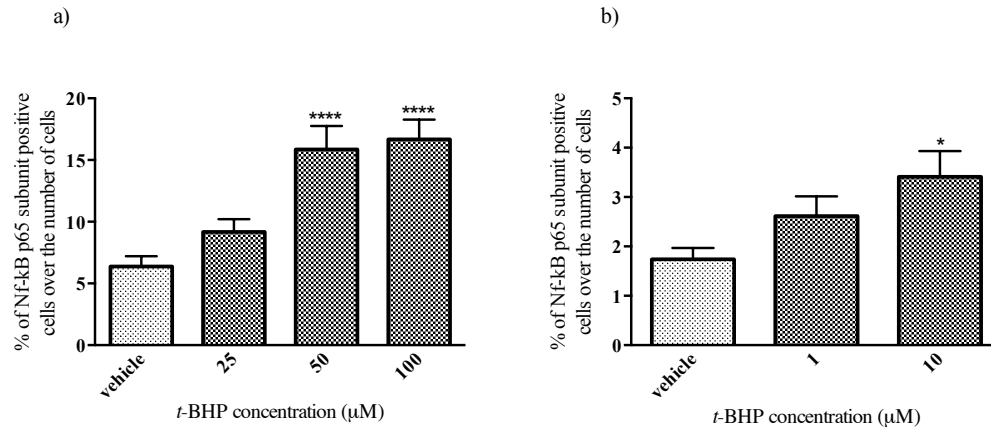


Figure 38. *t*-BHP treatment of the human hippocampal progenitor cells caused increase in the nuclear translocation of the NF-κB p65 subunit. a) A significant increase in the NF-κB p65 subunit nuclear translocation after 1 hour of *t*-BHP treatment was observed at 50μM (16%) and 100μM (17%) when compared to vehicle. **b)** A significant increase in the NF-κB p65 subunit nuclear translocation after 24 hours of *t*-BHP treatment was observed at 10μM (4%). Data are shown as mean ± SEM, *p<0.05, ****p<0.0001, compared with vehicle treatment. Three independent experiments were conducted on three independent cultures (n=3).

3.5 Oxidative stress induced by *t*-BHP in the human progenitor

hippocampal cells: in pursuit for inflammation

Many studies demonstrate increased biomarkers of OS and inflammation in depressed patients, however the underlying molecular mechanisms of the complex interplay of these systems remain to be unclear. Some of the proposed potential ways by which increased OS might trigger inflammatory cascades and vice versa are described in the introduction of this thesis, section 1.2.6. One of those mechanisms is a redox-induced activation of NF- κ B transcription factor, mostly associated with the immune response and propagation of pro-inflammatory cascades, despite the diversity of biological roles it fulfils. Previously it was reported that the human hippocampal progenitor cells I use in this thesis are immunocompetent, that is, they respond to, and produce, inflammatory molecules (Horowitz et al., 2015). Immunocompetent cells respond to inflammatory stimulation by activation of intracellular signalling mechanisms, leading to the transcription of a number of cytokines and their secretion from the cell. In particular, stimulation with IL-1 β leads to the secretion of a number of pro-inflammatory cytokines including IL-6 (Horowitz et al., 2015), that is routinely found increased in the blood of depressed patients along with other cytokines, and have been implicated in the pathogenesis of depression (Howren et al., 2009; Dowlati et al., 2010; Jansen et al., 2015). This inflammatory stimulation in response to IL-1 β was accompanied by activation of NF- κ B, which I found to be significantly increased in cells treated with 50 μ M and 100 μ M of *t*-BHP. Thus, I sought to investigate whether the peroxide lead to increased levels of cytokines, indicative of an activated immune response.

3.5.1 *t*-BHP treatment does not lead to increased secretion of cytokines

In order to measure the levels of cytokines produced by the cells upon OS insult I used two different methods: I performed an ELISA assay to detect IL-6 using the human IL-6 Quantikine ELISA kit (R&D Systems) and I conducted a multiplex protein quantification with Luminex platform using the Human Cytokine Magnetic 25-Plex Panel (Invitrogen). Cells were treated in 96-well plates with a range of concentrations of *t*-BHP from 1 μ M to 1mM for 1 hour. The supernatant was collected and stored at -80°C for further analysis, cells were washed and fresh media was added for another 23 hours and the supernatant was collected again and stored at -80°C before the assay performance. Both supernatants collected after one hour and after subsequent 23 hours were analysed. The concentration of all of the cytokines across all samples was either below the detection range or outside of the standard curve and therefore not qualified for analysis. A representative example of the standard curve and pg/ml concentrations of IL-6 in the samples treated with *t*-BHP derived from the known standards are presented in **Figure 39** and **Table 10** below.

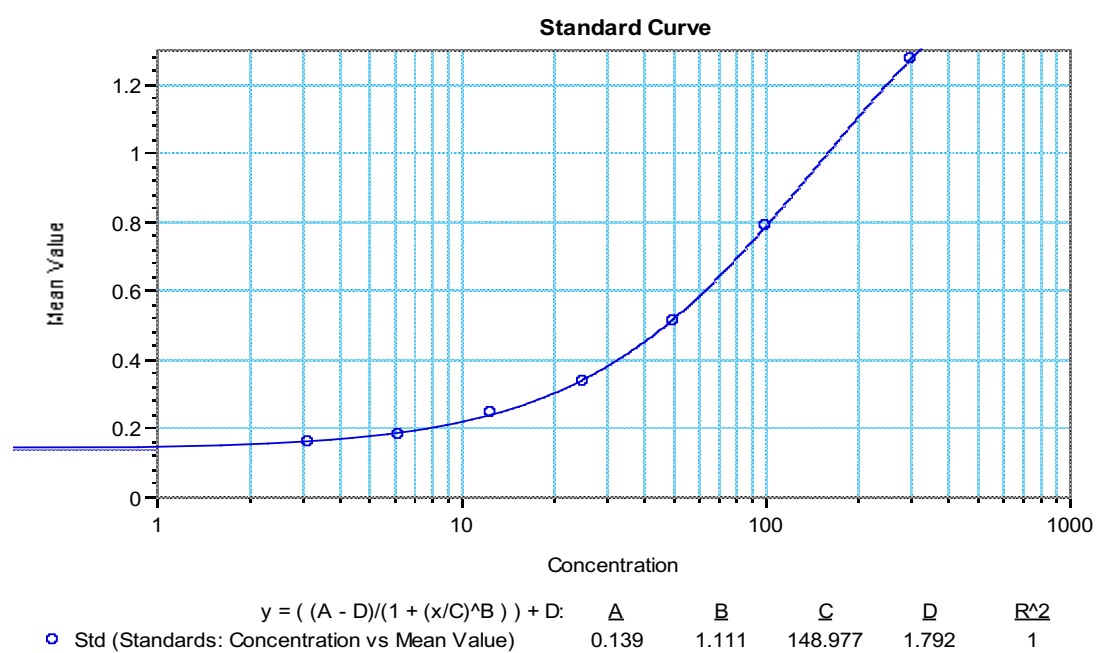


Figure 39. Standard curve based on the results from the provided standards in IL-6 Quantikine ELISA kit (R&D Systems) generated using SoftMax Pro (Molecular Devices).

<i>t</i> -BHP concentration	vehicle	1 μ M	10 μ M	25 μ M	50 μ M	100 μ M	200 μ M	500 μ M	1000 μ M
1 st repeat	0.033	0.01	0.005	below range	0.028	0.014	0.001	below range	0.032
2 nd repeat	0.006	0.061	0.167	0.067	0.031	0.041	below range	0.152	below range
3 rd repeat	0.024	0.805	0.124	0.056	below range	0.001	0.156	below range	0.061

Table 10. Concentrations of IL-6 in pg/ml in the supernatants collected from the human hippocampal progenitor cells treated with a range of concentrations of *t*-BHP after 1 hour. Data from three independent experiments conducted on three independent cell cultures presented as the mean of a duplicate of the sample in pg/ml of IL-6. Data analysed with a four parameter logistic algorithm to derive concentrations of the samples from known standards using SoftMax Pro (Molecular Devices).

3.5.2 *t*-BHP treatment did not affect the kynurenine pathway

Increased OS is associated with alterations in the kynurenine pathway that has been implicated in the pathogenesis of depression (Tan et al., 2012; Lovelace et al., 2016). I therefore wished to investigate whether *t*-BHP would lead to perturbations in the kynurenine pathway in the human hippocampal progenitor cells. Cells were treated in 96-well plates with 200µM, 500µM and 1000µM of *t*-BHP for 1 hour. The supernatant was collected and stored at -80°C, cells were washed and fresh media was added for another 23 hours and the supernatant was collected again and stored at -80°C. Both supernatants collected after one hour and after subsequent 23 hours were sent for HPLC analysis. Kynurenine (KYN), quinolinic acid (QUINA), tryptophan (TRP), anthranilic acid (ANA), 3-hydroxyanthranilic acid (3-HANA), kynurenic acid (KYNA) and 3-hydroxykynurenine (3-HK) were measured in the samples. QUINA, 3-HANA, KYNA and 3-HK were not detected. KYN, TRP and ANA were detected in all samples, however no significant differences across conditions were observed (**Figure 40**).

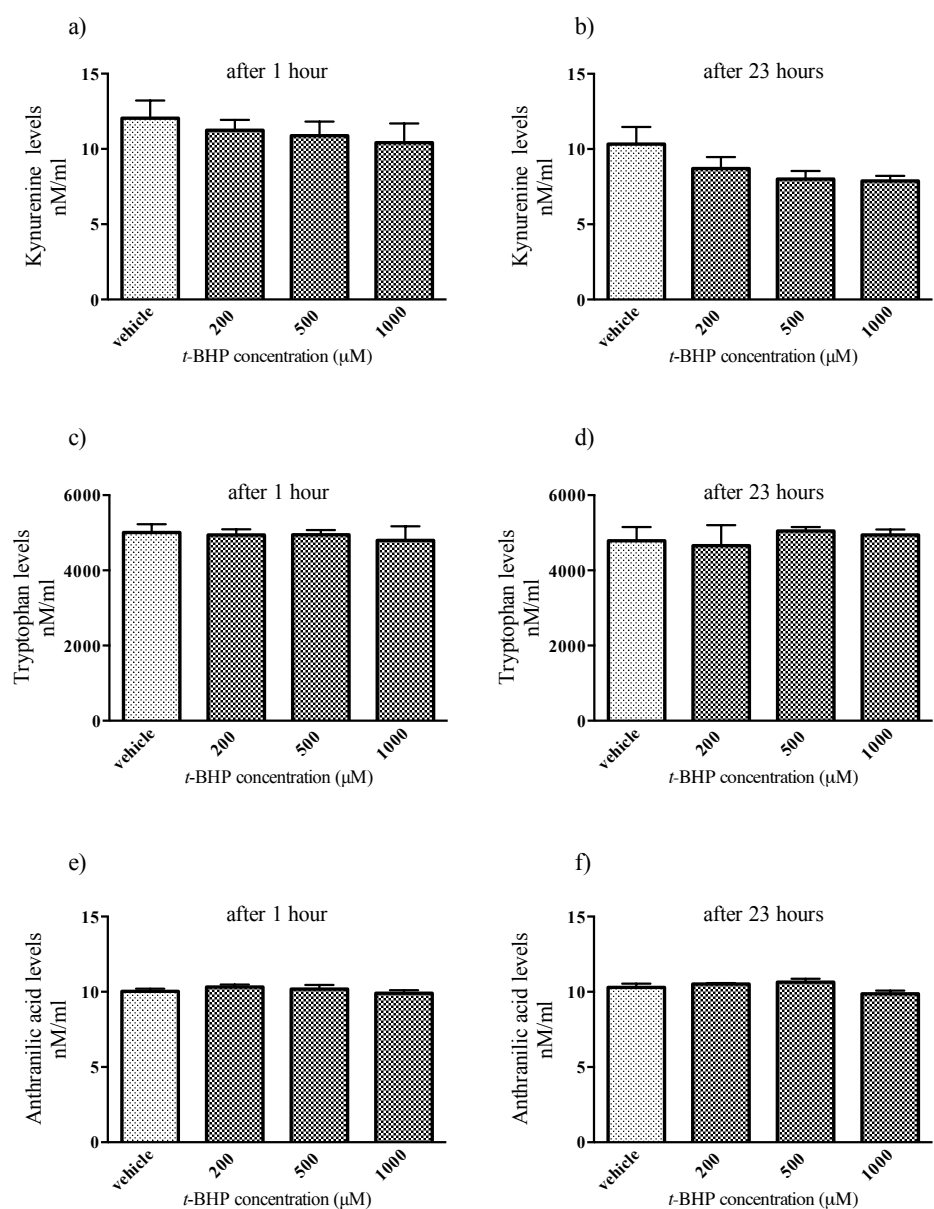


Figure 40. Metabolites of the kynurenine pathway detected in the supernatants of the human hippocampal progenitor cells treated with *t*-BHP. a, b) The concentration of kynurenine detected in the samples after 1 hour (a) and subsequent 23 hours (b) of *t*-BHP treatment; c, d) The concentration of tryptophan detected in the samples after 1 hour (c) and subsequent 23 hours (d) of *t*-BHP treatment; e, f) The concentration of anthranilic acid detected in the samples after 1 hour (e) and subsequent 23 hours (f) of *t*-BHP treatment. Three independent experiments were conducted on three independent cultures (n=3).

3.6 Testing various compounds with antidepressant action for the ability to prevent oxidative damage induced by *t*-BHP

In order to test whether various compounds with antidepressant action are capable of preventing the oxidative cell injury inflicted by *t*-BHP in the human hippocampal progenitor cells I used several paradigms including pre-treatment and co-treatment with these drugs before exposure to the peroxide. Two pre-treatment durations were implemented, of either 1 hour or 24 hours, while co-treatment of the compounds under investigation with *t*-BHP was always 1 hour. Based on the results of the previous experiments (section 3.1.1), two doses of *t*-BHP, 100 μ M and 500 μ M, were chosen in order to explore if the ability of antidepressant compounds to prevent oxidative cell damage depends on the severity of oxidative insult being applied. Following an oxidative injury in all experiments, cells were washed and cultured for the following 23 hours in fresh media. Subsequently cell viability assay was performed. In all experiments I included the following controls: vehicle conditions for the whole duration; exposure to culture media during the pre-treatment periods and treatment with either 100 μ M or 500 μ M of *t*-BHP only for 1 hour followed by 23 hours in fresh media.

3.6.1 Testing conventional antioxidants for the ability to prevent oxidative damage induced by *t*-BHP

I first sought to test the conventional antioxidants *N*-acetyl-cysteine (NAC) and GSH in order to validate the assay. First I tested the effect of NAC at 1mM and 10mM and 100mM on preventing the oxidative cell damage caused by *t*-BHP. This was the first time when NAC was used with HPC0A07/03C cell line in the OS conditions and

therefore, for screening purposes, I decided to use a range of NAC concentrations. I wanted to: 1) determine what would be the effective concentration of NAC to prevent OS damage in the cells and 2) if 1mM and 10mM of NAC were not effective, to ensure that the experimental protocol worked, since it was the first experiment to detect if prevention of the OS damage in the cells inflicted by *t*-BHP by antioxidants is possible. In this experiment treatment of the cells with 100 μ M and 500 μ M of *t*-BHP for 1 hour followed by 23 hours of culturing the cells in fresh media caused reductions in cell viability to 52%, $p<0.0001$ and 37%, $p<0.0001$ respectively. Pre-treatment of the cells with 1mM or 10mM of NAC for 1 hour followed by co-treatment of the cells with 1mM or 10mM of NAC plus either 100 μ M or 500 μ M of *t*-BHP for 1 hour did not have significant preventing effect on cell viability when compared to the cells treated with 100 μ M and 500 μ M of *t*-BHP only. In the same experimental design, but with 100mM of NAC cell viability reached 85%, $p<0.0001$ when co-treated with 100 μ M of *t*-BHP and 76%, $p<0.0001$ when co-treated with 500 μ M of *t*-BHP, being significantly different from the conditions where cells were treated with 100 μ M and 500 μ M of *t*-BHP only (**Figure 41**). The concentration of 100mM of NAC was not used in further experiments due to being physiologically irrelevant. In my following experiments, NAC was substituted by its cell permeable precursor GSH-monoethylester, where preventive effects on OS damage were achieved with lower doses, as described in the next section.

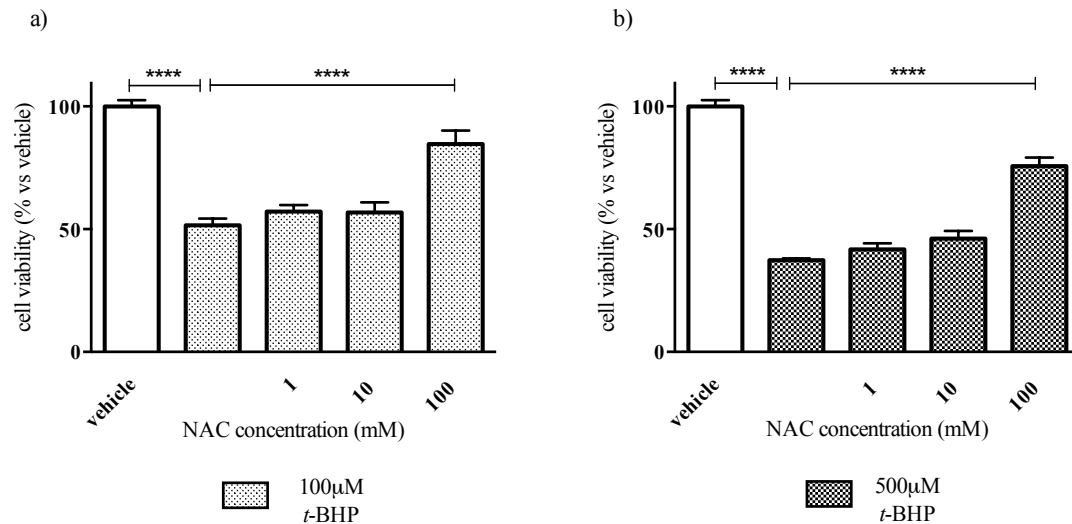


Figure 41. 1 hour pre-treatment and 1 hour co-treatment with 100mM of NAC plus *t*-BHP of the human hippocampal progenitor cells prevented reduction in cell viability caused by *t*-BHP. A significant reduction in cell viability was observed **a)** at 100µM of *t*-BHP (52%) and **b)** at 500µM of *t*-BHP (37%). **a)** Pre-treatment and co-treatment of the cells with 100mM of NAC plus 100µM of *t*-BHP abridged reduction in cell viability reaching 85%; **b)** 100mM of NAC plus 500µM of *t*-BHP abridged reduction in cell viability reaching 76%. Data are shown as mean \pm SEM, **** p <0.0001. Four independent experiments were conducted on four independent cultures (n=4).

Then I tested the effect of GSH at 1mM and 10mM on preventing the oxidative cell damage caused by *t*-BHP, implementing 24 hours of pre-treatment duration. In this experiment treatment of the cells with 100μM and 500μM of *t*-BHP for 1 hour followed by 23 hours of culturing cells in fresh media caused reduction in cell viability to 75%, $p<0.001$ and 46%, $p<0.0001$ respectively. Pre-treatment of the cells with 1mM or 10mM of GSH for 24 hours followed by co-treatment of the cells with 1mM or 10mM of GSH plus 100μM of *t*-BHP for 1 hour caused minor reduction in cell viability reaching 98%, $p<0.05$ and 91%, $p<0.01$ respectively, which presented a significant difference when compared to the cells treated with 100μM of *t*-BHP only. In the same experiment the same doses of GSH, but co-treated with 500μM of *t*-BHP caused reduction in cell viability to 76%, $p<0.0001$ at 1mM of GSH and 73%, $p<0.0001$ at 10mM of GSH, which was significantly different when compared to the cells treated with 500μM of *t*-BHP only (**Figure 42**).

There are various commercially available fluorometric kits for detecting intracellular GSH, however HPLC is a preferable method. GSH ethyl ester is a membrane/lipid permeable derivative of GSH which is effectively transported into the cells. For example, it was shown that preloading murine mesencephalic cultures for 24 h with 1-10mM GSH ethyl ester produced a dose-dependent increase in intracellular levels of GSH, reaching an increase of 191% when treated with 10mM of GSH ethyl ester compared with basal levels. (Zeevalk et al., 2007). My primary interest was to identify whether pre-treatment and co-treatment of the human hippocampal progenitor cells with GSH ethyl ester protected cells from *t*-BHP damage and thus I focused on the cell viability assay. However, it would be instructive to identify intracellular basal GSH levels and GSH levels upon treatment with GSH ethyl ester in the human hippocampal progenitor cells in future research.

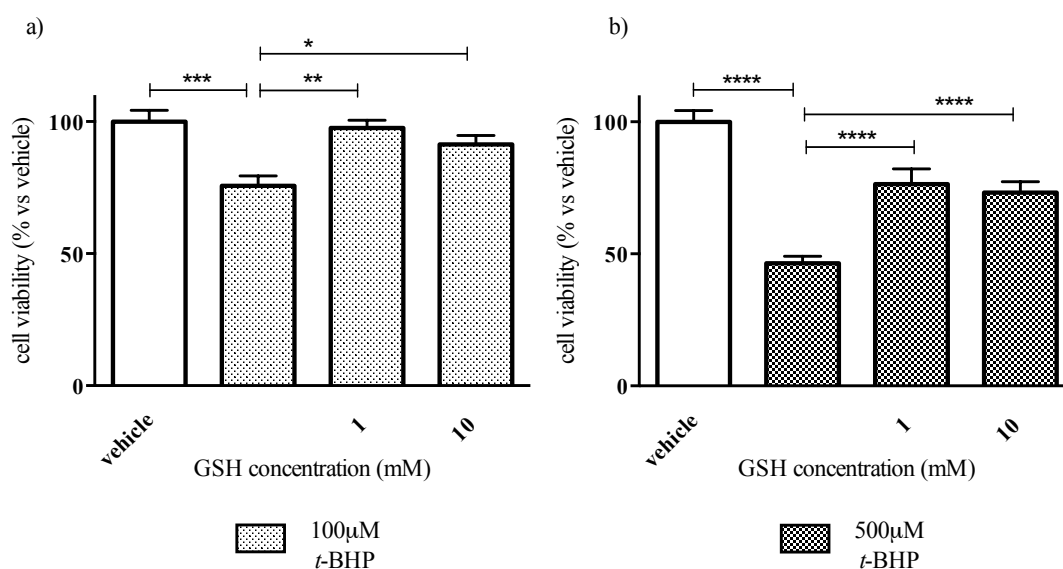


Figure 42. 24 hours pre-treatment and 1 hour co-treatment with GSH plus *t*-BHP followed by 23 hours of incubation in fresh media of the human hippocampal progenitor. A significant reduction in cell viability was observed **a)** at 100µM (75%) and **b)** at 500µM (46%). **a)** Pre-treatment and co-treatment of the cells with 1mM or 10mM of plus 100µM of *t*-BHP abridged reduction in cell viability reaching 98% and 91% respectively. **b)** Pre-treatment and co-treatment of the cells with 1mM or 10mM of GSH plus 500µM of *t*-BHP abridged reduction in cell viability reaching 76% and 73% respectively. Data are shown as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Four independent experiments were conducted on four independent cultures (n=4).

3.6.2 1 hour pre-treatment and 1 hour co-treatment with antidepressants plus *t*-BHP did not prevent the reduction in cell viability caused by oxidative insult

The commonly used SSRI antidepressant sertraline and the SNRI venlafaxine at concentrations 100nM and 1μM were tested in exactly the same experimental paradigm as described in the previous section. I chose these treatment doses based on previous *in vitro* research in our lab with this particular cell line (Anacker et al., 2011; Horowitz et al., 2015). No significant preventing effect of these drugs on cell viability when compared to the cells treated with 100μM and 500μM of *t*-BHP was observed (**Figure 43a, b, c, d**). I then tested the NMDA receptor antagonist ketamine, more recently being used as antidepressant, at two clinically relevant concentrations, 1μM and 10μM, similar to those detected in the plasma of depressed patients treated with ketamine (Zarate et al., 2012). No significant preventing effect of ketamine on cell viability when compared to the cells treated with 100μM and 500μM of *t*-BHP was observed (**Figure 43e, f**). Data is presented as the percentage of viable cells in treated cultures compared to those in control cultures.

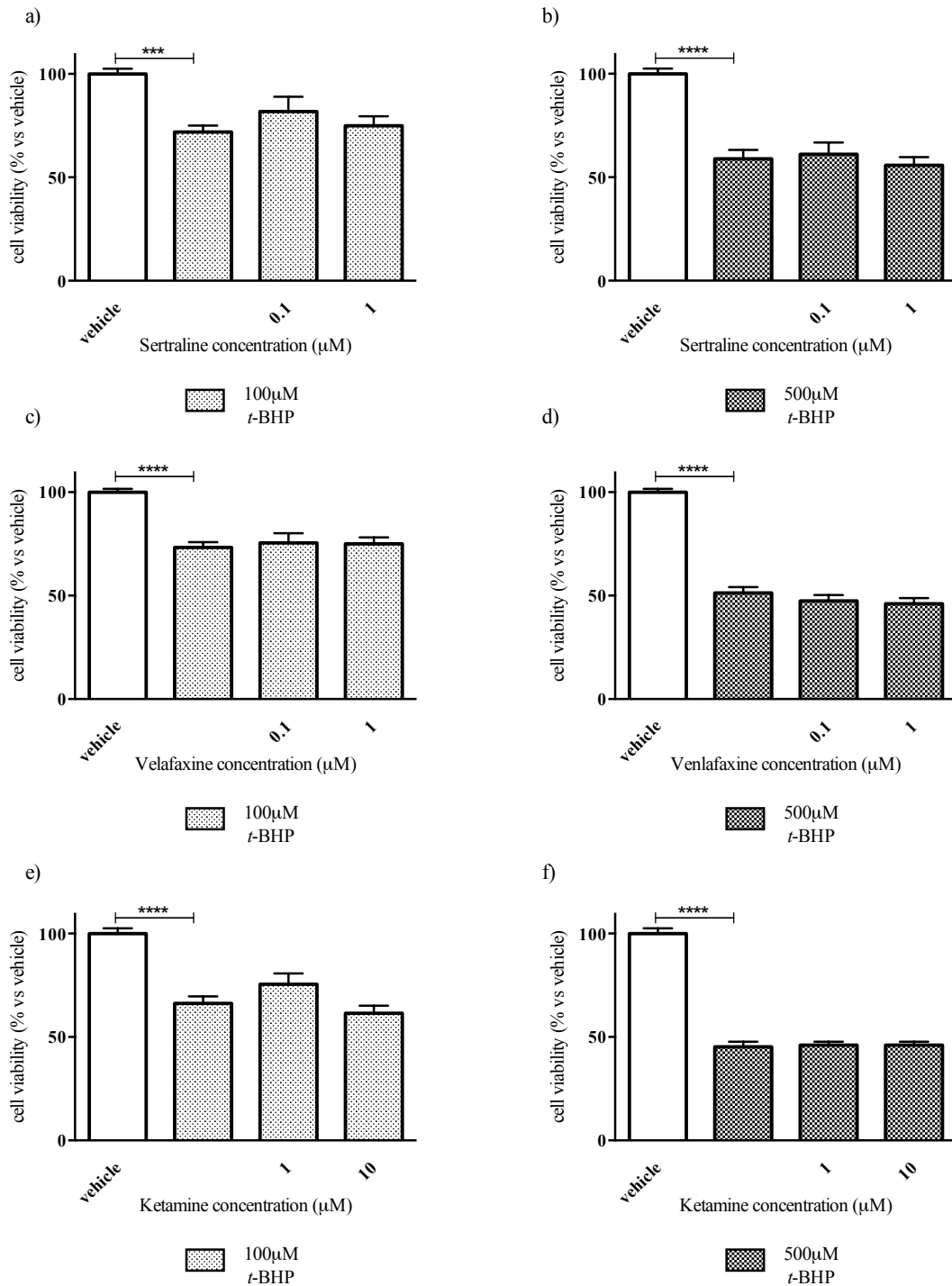


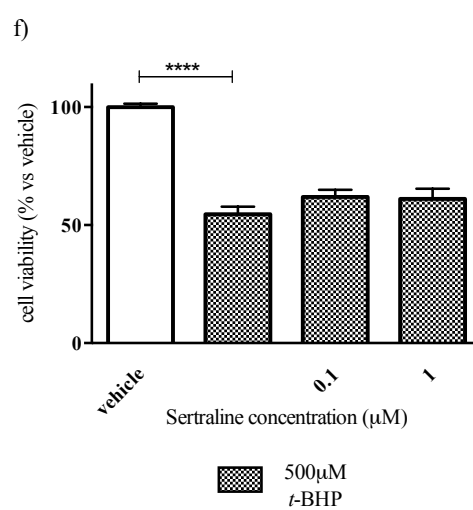
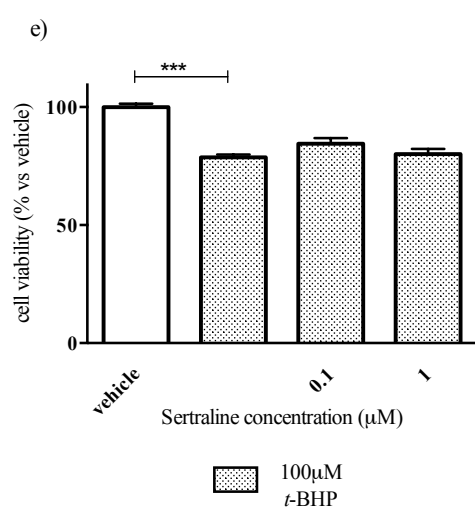
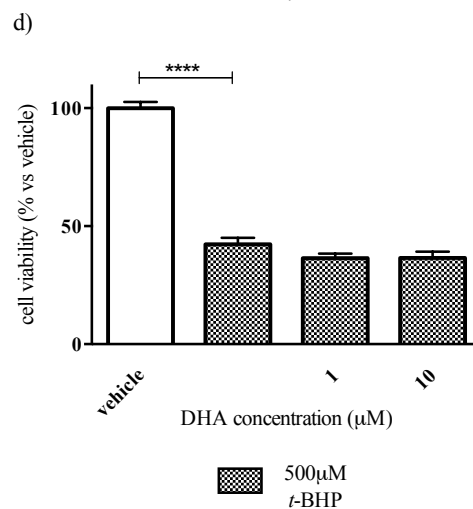
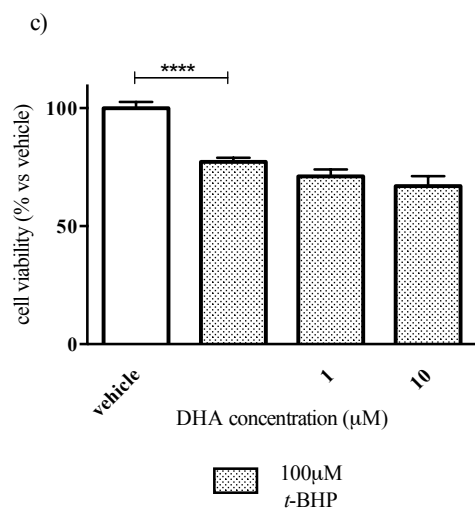
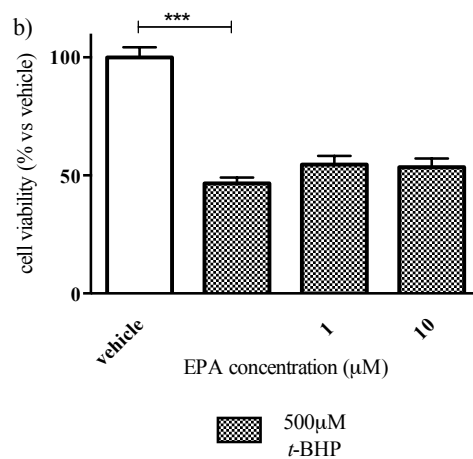
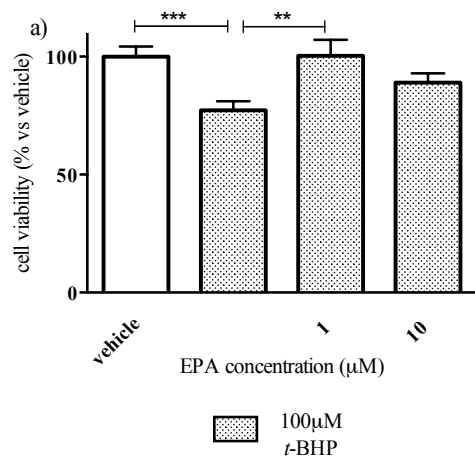
Figure 43. 1 hour pre-treatment and 1 hour co-treatment with antidepressant compounds plus *t*-BHP of the human hippocampal progenitor cells did not prevent reduction in cell viability caused by *t*-BHP. A significant reduction in cell viability was observed: **a)** at 100μM to 72%, **b)** at 500μM to 59%, **c)** at 100μM to 73%, **d)** at 500μM to 51%, **e)** at 100μM to 66%, **f)** at 500μM to 45%. No effect of

a,b) sertraline, **c,d)** venlafaxine and **e,f)** ketamine on prevention of reduction of cell viability inflicted by *t*-BHP when compared to the cells treated with 100μM or 500μM of *t*-BHP only was observed. Data are shown as mean ± SEM, *** $p < 0.001$, **** $p < 0.0001$. At least four independent experiments were conducted on at least four independent cultures (n = 4-6).

3.6.3 24 hours pre-treatment and 1 hour co-treatment with EPA plus *t*-BHP, but not antidepressants or DHA, prevented the reduction in cell viability caused by oxidative insult of the human hippocampal progenitor cells.

I hypothesized that a pre-treatment duration of 1 hour may be not sufficient for sertraline, venlafaxine and ketamine to develop the antioxidative effect and therefore I tested these drugs at the same concentrations, but changing the pre-treatment duration from 1 hour to 24 hours. I also tested the omega-3 fatty acids and tested of EPA and DHA at 1 μ M and 10 μ M in this experimental paradigm for their ability to prevent the oxidative cell damage. These treatment doses were also chosen based on previous *in vitro* research in our lab with this particular cell line (Horowitz et al., 2015).

Pre-treatment of the cells with 1 μ M, but not 10 μ M of EPA for 24 hours followed by co-treatment with 1 μ M of EPA plus 100 μ M of *t*-BHP for 1 hour and subsequent incubation in fresh media for 23 hours completely prevented the reduction in cell viability caused by 100 μ M of *t*-BHP (100%, $p < 0.01$). This was significantly different from the cells treated with 100 μ M of *t*-BHP, where cell viability decreased to 77%, $p < 0.001$ (**Figure 44a**). When cells were treated with 500 μ M of *t*-BHP, EPA was not able to prevent the reduction in cell viability to 46% caused by the peroxide, neither when used at 1 μ M nor at 10 μ M (**Figure 44b**). DHA, ketamine, sertraline and venlafaxine were also tested in exactly the same experimental paradigm at the doses previously evaluated, but they showed no significant preventing effect on cell viability when compared to the cells treated with 100 μ M and 500 μ M of *t*-BHP only (**Figure 44c, d, e, f, j**).



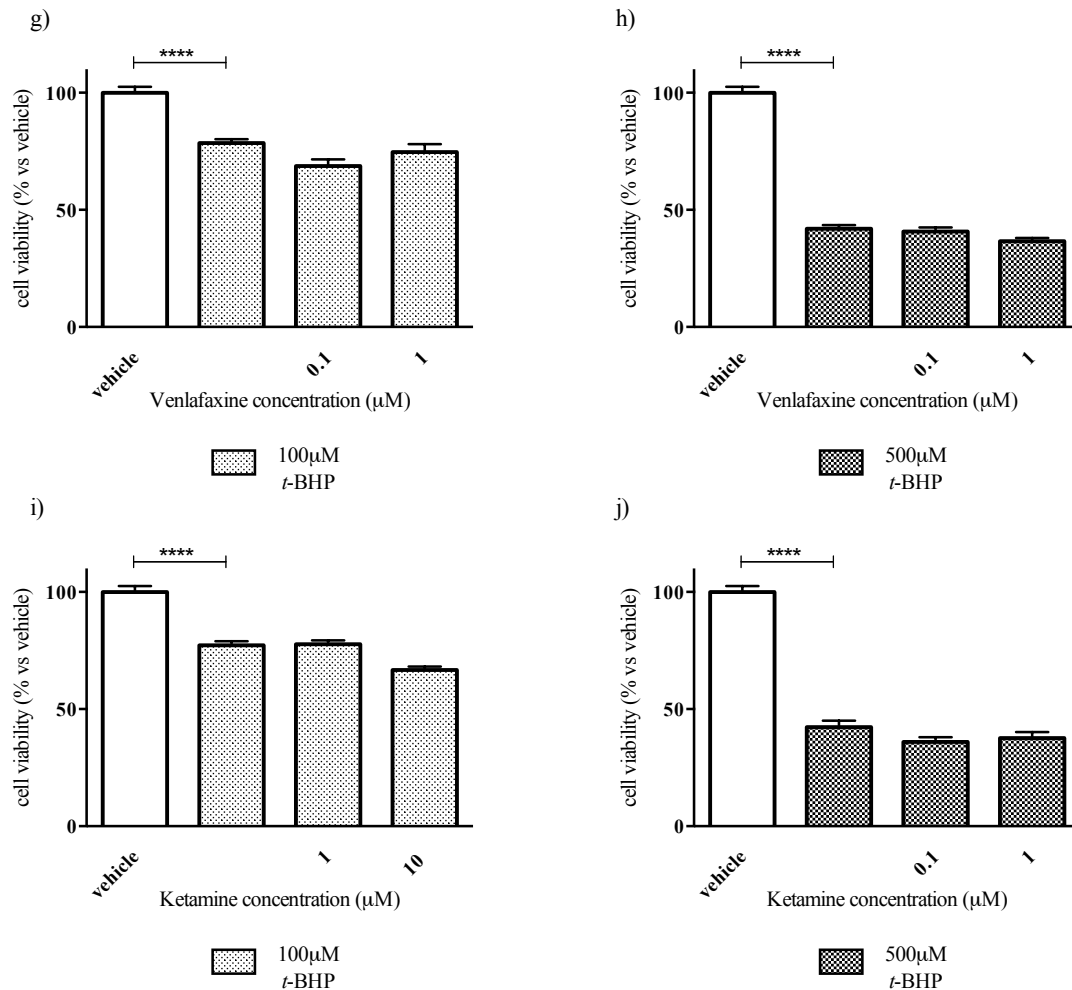


Figure 44. 24 hours pre-treatment and 1 hour co-treatment with antidepressant compounds plus *t*-BHP followed by 23 hours of incubation in fresh media of the human hippocampal progenitor cells. **a)** Pre-treatment and co-treatment of the cells with 1 μM of EPA prevented decrease in cell viability (100%) caused by 100 μM of *t*-BHP. A significant reduction in cell viability was observed, when cells were treated with *t*-BHP: **a)** at 100 μM to 77%, **b)** at 500 μM to 46%, **c)** at 100 μM to 77%, **d)** at 500 μM to 42%, **e)** at 100 μM to 78%, **f)** at 500 μM 56%, **g)** at 100 μM 78%, **h)** at 500 μM to 42%, **i)** at 100 μM to 77%, **j)** at 500 μM 42%. Data are shown as mean ± SEM, **p<0.01 ***p<0.001, ****p<0.0001. At least three independent experiments were conducted on at least three independent cultures (n = 3-6).

3.7 Modulation of gene expression by *t*-BHP and EPA and GSH in the presence of oxidative insult in the human hippocampal progenitor cells

In the previous section 3.7.2 I described that pre-treatment of the human hippocampal progenitor cells with 1 μ M of EPA prior to the 1 hour co-treatment with 100 μ M of *t*-BHP prevented the reduction in cell viability caused by the oxidative insult (**Figure 44a**). In order to further investigate potential molecular mechanisms that might underlie the antioxidative action of EPA, I sought to determine if EPA has any effect on antioxidative genes, so comparing it to the effects of *t*-BHP on its own in the same experimental set up. GSH, that had shown a significant preventive effect on the reduction in cell viability caused by *t*-BHP (**Figure 42**) was also included in the experiments in order to investigate its modulating effect on gene expression. Furthermore, I wanted to determine if there were any differential effects for EPA or GSH on gene expression. To obtain enough mRNA for gene expression analysis (**Table 6**) cells were grown in 6-well plates and the same experimental design as that used in the cell viability assays with a 24 hour pre-treatment period with the compounds under investigation was employed (**Figure 15c**). mRNA extraction was followed by RT-PCR and then qPCR was performed using commercially available TaqMan[®] assays for *HMOX1*, *NQO1*, *GCLM*, *Keap1*, *SLC7A11* and *SLC1A1* genes.

I hypothesised that EPA in the presence of an oxidative stimuli might lead to an increased activation of Nrf2, a major antioxidant transcription factor, through inhibiting the expression of *Keap1* and therefore, through regulation of synthesis of this protein. Keap1 is bound to Nrf2 in the cytoplasm and by that limits the nuclear translocation and activation of the transcription factor (**Figure 8**) (Itoh et al., 1999, 1997; Moi et al., 1994). I also wanted to determine if EPA pre-treatment leads to

upregulation of the antioxidative stress genes *HMOX1*, *NQO1*, *GCLM* and in particular *SLC7A11* in OS conditions. *SLC7A11* is the gene coding for the light chain xCT/SLC7A11 subunit of the system x_c⁻, the major provider of intracellular cysteine for GSH synthesis in exchange for one molecule of glutamate into the extracellular space (**Figure 6**). Since excessive extracellular glutamate is excitotoxic and has been implicated in the pathogenesis of depression (Mehta et al., 2013; Sanacora et al., 2012), I hypothesised that EPA pre-treatment of the cells in OS conditions might also lead to an enhanced glutamate clearance from the extracellular space through upregulation of the *SLC1A1* gene, coding for the glutamate transporter EAAT3.

3.7.1 Modulation of antioxidative stress genes by *t*-BHP and EPA and GSH in the presence of oxidative insult in the human hippocampal progenitor cells

Treatment of the human hippocampal progenitor cells with 100μM of *t*-BHP only caused a downregulation of *Keap1* of -20% (p<0.05); pre-treatment of the cells with 1μM of EPA and co-treatment with 100μM of *t*-BHP also caused a slightly higher downregulation of *Keap1* gene (-35%, p<0.01). Similarly, GSH caused a downregulation of *Keap1* gene of -47% (p<0.001) (**Figure 45a**).

Treatment of the cells with 100μM of *t*-BHP caused an upregulation of *HMOX1* gene of +64% (p<0.05), while pre-treatment of the cells with 1μM of EPA followed by co-treatment with 100μM of *t*-BHP caused a slightly higher upregulation of this gene of +92% (p<0.01). When cells were pre-treated with 1mM of GSH and co-treated with 100μM of *t*-BHP in the same experimental set up, no significant difference in *HMOX1* gene expression were observed when compared to vehicle (**Figure 45b**). No

significant difference in expression of *NQO1* or *GCLM* genes across conditions was observed when compared to vehicle (**Figure 45c, d**).

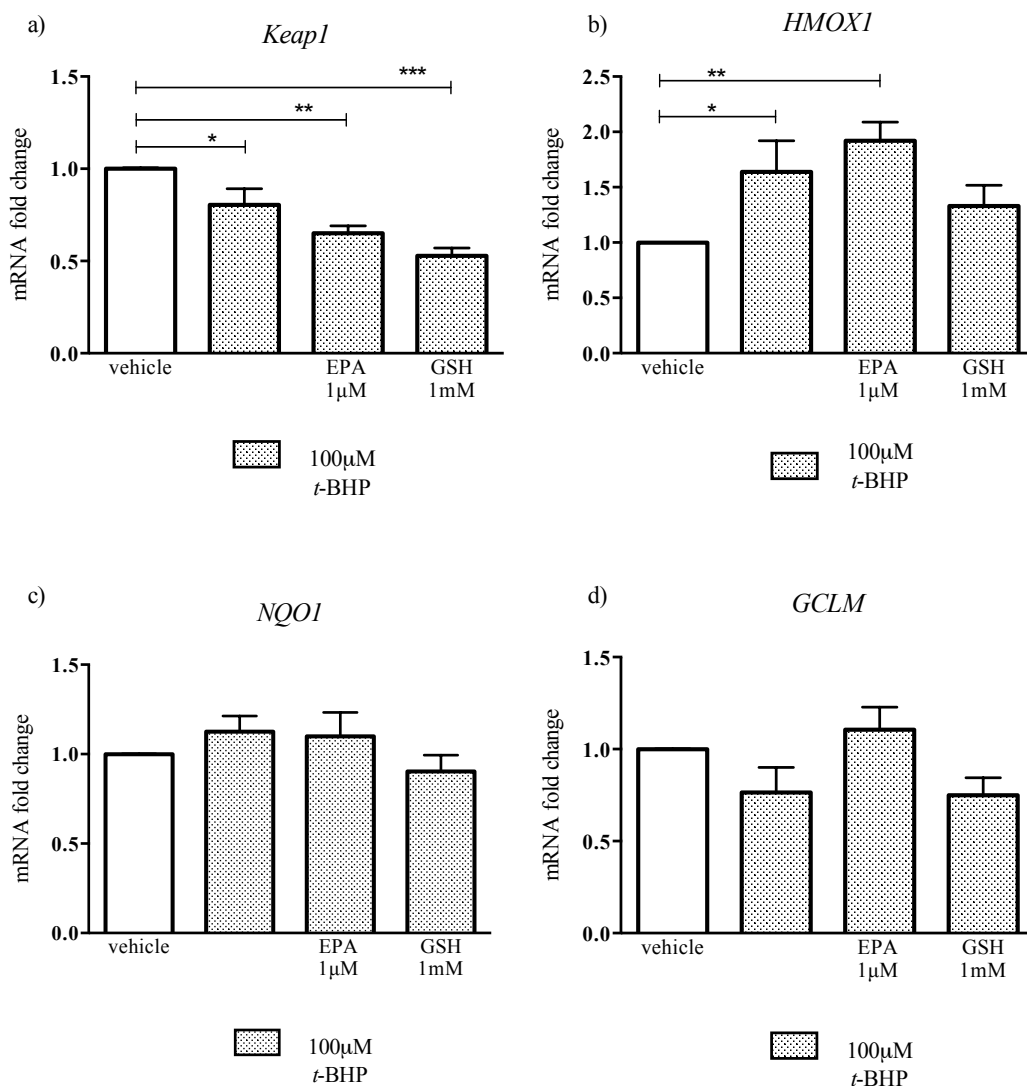


Figure 45. Modulation of antioxidative gene expression by *t*-BHP and EPA and GSH in the presence of *t*-BHP in the human hippocampal progenitor cells. Cells were pre-treated with the compound under investigation for 24 hours, then co-incubated with *t*-BHP and the compound under investigation for 1 hour followed by incubation in fresh media for 23 hours. **a)** A significant downregulation of *Keap1* was observed at 100μM of *t*-BHP (-20%), at 1μM of EPA plus at 100μM of *t*-BHP (-35)% and at 1mM of GSH plus at 100μM of *t*-BHP (-47%). **b)** A significant upregulation of *HMOX1* was observed at 100μM of *t*-BHP (+64%), at 1μM of EPA plus at 100μM of *t*-BHP (+92%). **c, d)** No significant difference was observed in expression of *NQO1* and *GCLM* gene across conditions when compared to vehicle. Data are shown as

mean \pm SEM, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. At least five independent experiments were conducted on at least five independent cultures (n=5-11).

3.7.2 Modulation of system x_c^- and glutamate transport by *t*-BHP and EPA and GSH in the presence of oxidative insult via gene expression regulation in the human hippocampal progenitor cells

Treatment of the human hippocampal progenitor cells with 100 μ M of *t*-BHP caused a downregulation of *SLC7A11* of -25% ($p < 0.05$). Pre-treatment of the cells with 1 μ M of EPA followed by co-treatment with 100 μ M of *t*-BHP prevented the downregulation of *SLC7A11* caused by the oxidative injury and was significantly different from the condition where cells were treated with the peroxide only, showing now an upregulation of +38% ($p < 0.01$). GSH did not have similar effects to EPA on the expression of *SLC7A11*, and this condition was not different from the cells treated with 100 μ M of *t*-BHP only, but was significantly different from vehicle, showing a -42% ($p < 0.001$) decrease (**Figure 46a**). It is possible that in this case the pre-treatment of cells with GSH ethyl ester had limited effects because significant increases in intracellular GSH levels were not achieved. Interestingly, it was demonstrated in murine mesencephalic cultures that the half-life for clearance of loaded 5mM of GSH ethyl ester for 24 hours which was then switched to fresh media was ~10 hours and ~14 hours in normal and GSH-depleted cultures respectively (Zeevalk et al., 2007). This issue can be addressed in the human hippocampal progenitor cells in further research by performing assays to detect the intracellular GSH levels.

Treatment of the cells with 100 μ M of *t*-BHP or pre-treatment with 1 μ M of EPA followed by co-treatment with EPA and the peroxide did not cause significant changes in the levels of expression of *SLC1A1*. Pre-treatment of the cells with 1mM of GSH and co-treatment with 100 μ M of *t*-BHP led to a downregulation of *SLC1A1* gene of -35% ($p < 0.01$) when compared to vehicle (**Figure 46b**).

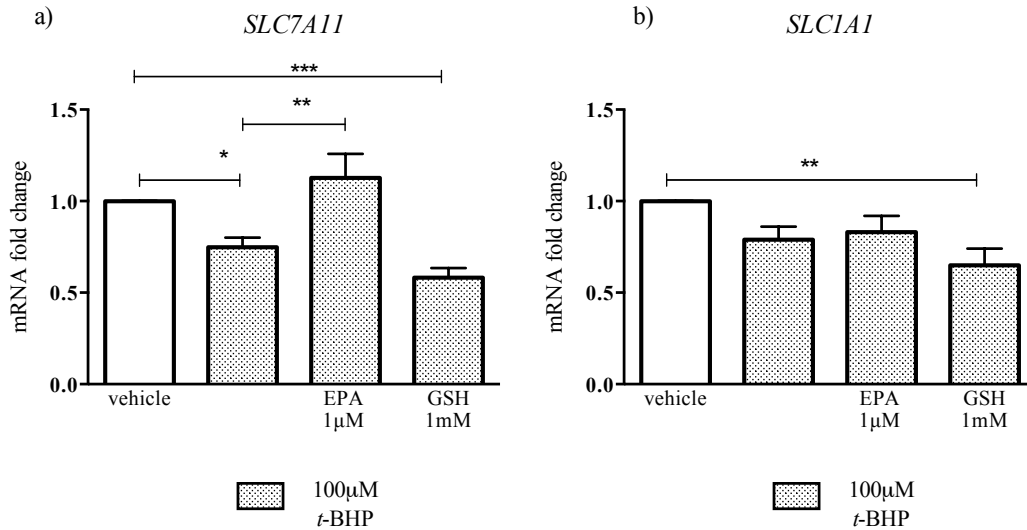


Figure 46. Modulation of *SLC7A11* and *SLC1A1* genes by *t*-BHP and EPA and GSH in the presence of oxidative in the human hippocampal progenitor cells.

Cells were pre-treated with the compound under investigation for 24 hours, then co-incubated with *t*-BHP and the compound under investigation for 1 hour followed by incubation in fresh media for 23 hours. **a)** A significant downregulation of *SLC7A11* was observed at 100µM of *t*-BHP (-25%). 1µM of EPA plus 100µM of *t*-BHP upregulated *SLC7A11* (-38%) compared to 100µM of *t*-BHP only. 1mM of GSH plus 100µM of *t*-BHP downregulated *SLC7A11* (-48%) compared to vehicle. **b)** 1mM of GSH plus 100µM of *t*-BHP downregulated *SLC1A1* (-35%) compared to vehicle. Data are shown as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$. At least five independent experiments were conducted on at least five independent cultures (n=5-9).

4 Discussion

4.1 Summary of findings

In this thesis I have set up an experimental *in vitro* model of OS in human hippocampal cells, using the progenitor cell line HPC0A07/03C capable of neurogenesis. I have studied potential molecular mechanisms thought to be involved in the pathogenesis of MDD, as well as tested the ability of various compounds with antidepressant action to prevent detrimental changes caused by oxidative damage. I have demonstrated that *t*-BHP-induced OS caused significant reductions in cell viability and an increase in lipid peroxidation, indicative of oxidative damage, in a dose-dependent manner, with no difference in the levels of CC3, an apoptotic marker. The omega-3 fatty acid EPA and the conventional antioxidants NAC and GSH prevented the inflicted oxidative damage caused by *t*-BHP, by modulating the expression of a series of genes involved in the OS response. Neither DHA nor sertraline, venlafaxine or ketamine showed similar cytoprotective effects against OS in my model. Additionally, I investigated the impact of alterations in redox homeostasis on cellular neurogenesis and found that treatment of the cells with low doses of *t*-BHP during proliferation and differentiation lead to major changes in neurogenesis – an increase in neuroblasts and mature neurons. I also identified potential redox-regulated signal transduction pathways involved in these processes by studying the changes in the activity of the family of MAPKs. Blocking ERK1/2 activation abrogated the peroxide-induced differentiation of the progenitor cells into mature neurons. Treatment with *t*-BHP activated the redox-sensitive transcription factor Nrf2, which plays a major role in antioxidant defence. Similarly, *t*-BHP also

activated NF- κ B, a known regulator of immune responses, but with no signs of inflammation in the human hippocampal progenitor cells.

The findings in this thesis extend our current understanding of redox-related molecular mechanisms considered to underlie the pathophysiology of MDD and provide confirmation for the omega-3 fatty acid EPA, known to possess antidepressant properties, to have antioxidant characteristics, identified on cellular and gene expression levels. The individual findings of this thesis are summarised below:

4.1.1 Aim 1

The first aim of this thesis was to establish a controlled and robust model of OS in the human hippocampal progenitor cells using *t*-BHP. I first identified the doses of *t*-BHP that cause significant cell damage by assessing cell viability using the MTS assay. Viability was assessed after 24 hours, both upon a continuous exposure of cells to the peroxide or upon an acute peroxide treatment of 1 hour followed by a change to fresh media. This last paradigm was chosen for my subsequent experiments as I wished to determine if cells survived after a certain period subsequent to the inflicted OS insult. To study preventive effects I selected treatments with *t*-BHP at 100 μ M and 500 μ M, which caused viability reductions to 71% and 28% respectively, and I decided to focus on concentrations below 100 μ M, that did not affect viability, to research cell signalling pathways, as slight redox shifts in the system towards an oxidised environment are expected to upregulate cellular defences and influence the cell fate. The oxidative damage was confirmed by an increase in levels of lipid peroxidation.

There were no significant differences in the apoptotic marker CC3, suggesting that cell death occurs through caspase-3 independent mechanisms.

4.1.2 Aim 2

The second aim of my thesis was to investigate how neurogenesis is affected in the human hippocampal progenitor cell line upon redox shifts and to identify potential underlying signalling pathways. Treatment with *t*-BHP caused a decrease in proliferation at doses of 25µM and 50µM, and an increase in the amount of Dcx-positive neuroblasts and MAP2-positive neurons at doses of 1µM and 10µM. Blocking ERK1/2 activation with the selective inhibitor FR180204 abrogated the redox-induced increase in mature neurons.

4.1.3 Aim 3

Aim 3 of this thesis was to gain mechanistic insights related to the pathogenesis of depression by investigating the interaction of redox and inflammatory systems. The transcription factors NF-κB and Nrf2 were activated by *t*-BHP treatment. Contrary to my hypothesis no cytokine secretion, which would be indicative of immune response in the cells, was detected in the supernatant. Treatment with *t*-BHP modulated antioxidative genes, causing an upregulation of *HMOX1*, coding for the enzyme heme oxygenase 1 that catalyzes the degradation of protein-unbound heme. The peroxide also caused a downregulation of *SLC7A11*, coding for the xCT subunit of system x_c⁻ regulating glutamate release in exchange for cystine, and of *Keap1*, coding for the protein that anchors Nrf2 to cytoplasm and thus keeps it inactivated. No changes were

observed in the expression of *NQO1*, coding for the enzyme that catalyzes the reduction of highly reactive quinones, *GCLM*, coding for a subunit of the rate-limiting enzyme in the synthesis of GSH, or *SLC1A1*, coding for the excitatory amino-acid transporter 3 (EAAT3), which plays an essential role in transporting glutamate across plasma membranes in neurons.

4.1.4 Aim 4

Finally, aim 4 of this thesis was to compare the effects of compounds with antidepressant action versus traditional antioxidants on the prevention of oxidative damage inflicted to the cells. Pre-treatment with NAC for a period of 1 hour or with GSH and EPA for 24 hours prevented the oxidative cell injury inflicted by *t*-BHP. Neither sertraline, venlafaxine, ketamine nor DHA showed any significant effect on prevention of oxidative damage in my *in vitro* model. EPA in the presence of *t*-BHP downregulated the expression of *Keap1* and upregulated *HMOX1* and *SLC7A11*, all genes involved in the OS response, potentially enhancing GSH production and restoring the *t*-BHP disturbed redox equilibrium. GSH in the presence of the oxidative stimulus downregulated *Keap1*, *SLC7A11* and *SLC1A1*, possibly to hinder further GSH synthesis.

4.2 Damaging effects of induced OS on the human hippocampal progenitor cells

4.2.1 Establishing the model

In order to establish a controlled and robust model of OS in the human hippocampal progenitor cells I had first to identify an appropriate OS-inducing compound, choose a reliable method for assessing the inflicted damage and then determine the concentration of the chemical to use and the duration of the treatment.

Although one might argue that the use of hydrogen peroxide is more physiologically relevant, *t*-BHP was chosen as the compound to induce OS *in vitro* model due to its higher stability. Hydrogen peroxide is unstable. A detailed study that assessed factors affecting the decomposition of this peroxide has shown that an increase in the concentration of copper ions, the presence of solids and changes in pH and temperature contribute to its loss in solution. In particular, a temperature-depending decrease in concentration was demonstrated when the temperature increased from 20°C to 50°C over a period of 3 hours, reaching 80% loss at 50°C (Yazici and Deveci, 2010). Given that the cells are cultured in incubators at 37°C to resemble physiological conditions, and considering that all the reagents used need to be pre-warmed in a water bath at 37°C, it seemed sensible to avoid H₂O₂ and use *t*-BHP instead, which was demonstrated to start undergoing thermolysis at 69,5° (Liu et al., 2012) – a temperature that is never reached in cell culture. *t*-BHP is a membrane permeable oxidant that has been previously used successfully to generate OS damage in various *in vitro* models. It is easily metabolized and can cause a variety of changes in biological and biochemical systems, such as generation of ROS, peroxidation of lipids, permeabilization of cell membranes, impairment of ATP synthesis, DNA

damage via radical and non-radical mechanisms and numerous effects on the antioxidant defence system (Chen and Chen, 2011).

There is a variety of commercially available assays that can be used to measure oxidative damage of cell constituents *in vitro*. The most well-established and frequently used indicators of oxidative damage are: a) 8-OHdG and 8-OHG that are generated upon DNA and RNA oxidation; b) protein carbonyl groups, that are produced by carbonylation of proteins; and c) 4-HNE and MDA that are formed upon lipid peroxidation. In particular, these biomarkers can be quantified using ELISA kits, HPLC or immunostaining using relevant antibodies. Often, multiple assays are required to assess oxidative damage of different cell structures. However, my primary interest was to identify whether and to what extent *t*-BHP treatment caused a decrease in cell viability of the human hippocampal progenitor cells. To confirm that *t*-BHP initiated oxidative damage I have chosen to measure the level of lipid peroxidation in the samples using TBARS assay (described in the next section), hypothesizing that lipids would be affected by the peroxide the most due being abundant in the cell membranes and thus being exposed to the treatment to a greater extent. It would be instructive to detect levels of protein and nucleic acids oxidative damage in my cell model in future research, to then further identify which particular molecules are mostly protected by particular antioxidative compounds.

In order to determine the toxic concentrations of *t*-BHP, I chose MTS, a colorimetric method that allows to estimate the amount of viable cells. This method has been widely used in *in vitro* studies to assess cytotoxicity of various compounds. Solid evidence that the MTS assay is a reliable test system of high accuracy and reproducibility comes from a comprehensive study that tested the cytotoxicity of numerous chemicals in two human epithelial cell lines to determine the sensitivity and

specificity of the assay. This study revealed that the influence of different operators, differences in cell number or tests on different occasions on the accuracy of the results is negligible (Malich et al., 1997).

In vitro studies have shown a huge variation in both the concentration of substances used to generate OS and the duration of treatment. Cells of different origin have been treated for periods ranging from 15 minutes to 24 hours, and at peroxide concentrations between nanomolar and millimolar (Bae et al., 2010; Harvey et al., 2012; Saffari and Sadrzadeh, 2004; Sée and Loeffler, 2001; Sun et al., 2010). This is not surprising, since the effects of induced OS on cells very much depend on the type of cells, the peroxide and the experimental conditions, and therefore the concentration and duration of treatments always needs to be selected singularly. Thus, in this thesis cells were treated with a range of *t*-BHP concentrations initially for 24 hours. No reduction in cell viability was observed at concentrations ranging from 1µM to 10µM, whereas a decrease in cell viability to 65% occurred at 25µM of *t*-BHP. This reduction increased to 30% at 50µM, reaching only 5% at 75µM and higher doses of *t*-BHP. Given such a pronounced decrease in cell viability between 25µM and 50µM and considering the immense cell death due to the massive oxidative damage inflicted by the peroxide already at 75µM of *t*-BHP I changed the experimental design, modifying the time of peroxide treatment to 1 hour. I hypothesized that the longer the duration of *t*-BHP exposure the lower is the toxic dose, suggesting the occurrence of a cumulative cytotoxic effect. Therefore, I decided to decrease the time of exposure and additionally to determine if cells survived after a certain period subsequent to the inflicted OS insult. Thus, a variation in the experimental set-up was applied, treating cells acutely for 1 hour followed by 23 hours of culturing in fresh media and keeping the same total culture time before the MTS assay was performed (so 24 hours in

total). The difference in the paradigms was that in my first one I was identifying cell damage straight after the OS insult, in the second one I determined if cells survived after an oxidative challenge, when the stimulus is no longer applied. This last acute 1 hour treatment with *t*-BHP followed by 23 hours of culturing in fresh media caused a significant delayed decrease in cell viability in a dose-dependent manner to 71% at 100 μ M, 42% at 200 μ M, 28% at 500 μ M and 14% at 1mM. Exposure times greater than 24 hours are considered to be logistically difficult in an early screening paradigm, while exposures of less than 24 hours may not allow enough time for toxicity to occur, a situation that could produce a large number of false negative results (McKim, 2010). After removal of the peroxide upon a 1 hour treatment, the toxic effect can expand due to chain oxidation reactions initiated by *t*-BHP, or by damaged cells spreading injury to the surrounding “intact” cells by releasing metals (Halliwell and Gutteridge, 2015). Treatment of the cells for 1 hour was chosen for all subsequent experiments due to a pronounced concentration response curve observed in the cell viability experiments. Endpoints obtained after 1 hour of peroxide exposure provided a platform for assessment of the cytoprotective effects of the compounds under investigation, choosing two doses of *t*-BHP to estimate if the potential antioxidative effect depends on the OS level.

A significant reduction in the total number of cells was observed by direct counting of DAPI-positive cells at doses of *t*-BHP starting at 10 μ M, suggesting that there is some toxicity effect at lower levels of OS insult. Fixing the cells with PFA in preparation for immunocytochemistry allows to obtain only a snapshot of a certain time point. Furthermore, PFA cells fixing is a harsh procedure where cells are also subjected to a number of washes which contributes to the existing OS insult, and damaged cells are likely to be rinsed away, while in a cell viability assay they would contribute to the

signal. Thus, it would not be apparent from these data whether the toxic effects were due to a direct OS insult or a cumulative effect of OS, or due to PFA and the fixing procedure. While immunocytochemistry estimates the total number of cells, the MTS assay measures their metabolic capacity and the data represent an average of the signal from the total cell population. In this viability assay cells are allowed to metabolise the tetrazolium compound added to the culture media into the colorimetrically detectable formazan product. Damaged and apoptotic cells still remain metabolically active and produce formazan. Therefore, I chose the MTS assay and selected the cytotoxicity doses of *t*-BHP obtained with this method for further experiments rather than assessing the most resilient cells that survived after both the OS and PFA challenges, as having cells damaged to different extent by OS, which also contribute to physiological or pathological processes, is more representative of an *in vivo* situation.

As a result, a paradigm of 1 hour treatment with 100 μ M and 500 μ M of *t*-BHP followed by 23 hours of culturing in fresh media has been chosen to induce OS and to test compounds for their ability to prevent oxidative damage. I also wanted to examine how redox shifts in my *in vitro* model might affect cell signalling pathways. Considering that high levels of OS cause severe damage, with failure to activate protective mechanisms and halted cell cycles, concentrations of *t*-BHP below 100 μ M, where no reduction in cell viability was observed, were used in experiments investigating redox signal transduction pathways, where a moderate and mildly oxidised environment is expected to lead to activation of transcription factors, upregulation of antioxidative defences and promotion of cellular proliferation or/and differentiation.

4.2.2 Lipid peroxidation

In my experiments a significant increase in the level of lipid peroxidation, measured with the TBARS assay, was observed in cells treated with 500 μ M and 1000 μ M of *t*-BHP after 1 hour, although a clear trend was already observed at 50 μ M, 100 μ M and 200 μ M of *t*-BHP. *t*-BHP is a strong free radical source and has been particularly utilized to induce lipid peroxidation both *in vivo* (Resende et al., 2008) and *in vitro* (Ayala et al., 2014; Guo et al., 2016; Pavlica and Gebhardt, 2010) in studies modelling OS.

End products of lipid peroxidation are ubiquitously generated in biological systems and their effects are particularly important in post-mitotic cells that gradually accumulate oxidative damage over time, such as neurons. In particular, lipid peroxidation product accumulation in human tissues is a major cause of cellular dysfunction playing a major role in neurodegenerative disorders. OS induces peroxidation of the cellular membrane lipids and circulating lipoprotein molecules generating highly reactive aldehydes, that are much more stable than ROS and are capable of spreading from their site of origin and act at more distant sites. Thus, these carbonyl compounds can be more destructive than ROS and may have far-reaching damaging effects on target sites within or outside membranes, as they can react with nucleophilic groups in macromolecules like proteins and DNA (Negre-Salvayre et al., 2010). One of these products is MDA, a membrane-permeable end-product generated by decomposition of arachidonic acid and larger PUFAs, exhibiting various biological functions, such as regulating gene expression or modifying DNA bases.

In order to confirm any oxidative damage in the human hippocampal cells and to identify whether *t*-BHP treatment of the cells leads to an increase in lipid peroxidation I used the TBARS assay, which is commonly reported as a measure of MDA across

studies (Guo et al., 2016; Pavlica and Gebhardt, 2010). Although it is believed that TBARS assay measures MDA in the sample, in fact in complex biological systems, many compounds (including simple and complex carbohydrates, protein oxidation products, and nucleic acid oxidation products) react with thiobarbituric acid and produce coloured adducts (Forman et al., 2015). Moreover, more free radicals are generated during the assay procedure which leads to oxidation of more molecules and lipids and hence more MDA. Additionally, there are other several compounds other than MDA (such as streptomycin, sialic acid and biliverdin) that react in the TBA assay and absorb at the same wavelength when analysed with a plate reader. These problems can be overcome by using HPLC, where (TBA)₂-MDA adducts can be separated from other chromogens. By use of this method, picomole quantities of MDA can be readily and specifically detected in different biological materials (Khoschsorur et al., 2000). However, this technique also has limitations, often is not available and is more complex. Irrespective of whether the assay detects only MDA or not, in my experiments a clear dose response curve was observed. MDA is the major component reacting with thiobarbituric acid. MDA has been widely used as a biomarker for lipid peroxidation in clinical studies and is contently found to be increased in depressed patients. In a recent meta-analysis that demonstrated greater lipid peroxidation in MDD than in controls 12 out of 17 studies included used MDA as a biomarker (Mazereeuw et al., 2015). Hence, increased levels of MDA observed in depressed patients are resembled in my *in vitro* OS model for studying molecular mechanisms of the MDD pathogenesis.

4.2.3 Cleaved Caspase-3—independent cell death

In this thesis I examined whether *t*-BHP–induced death of the human hippocampal progenitor cells is characterised by changes in expression of the marker cleaved caspase-3 (CC3), a hallmark of apoptosis. In my experiments, the oxidative insults did not cause an increase in CC3 expression, suggesting that cell death occurred through caspase-independent mechanisms.

Neuronal cell death is regulated by multiple interconnected signalling pathways. The most distinct forms of cell death are apoptosis and necrosis. Conventionally, apoptosis was considered to be a genetically programmed physiological cell death, regulated by enzymatic cascades during development, homeostasis, infection and pathogenesis, where an organized degradation of the cell occurs within an intact plasma membrane. CC3 is a critical executioner of this type of cell death and is also a feature of many chronic neurodegenerative diseases (D'Amelio et al., 2012). Necrosis was mostly considered to be a cell death that occurs in response to physicochemical insults and is associated with cell swelling, gross membrane damage and leakage of cell constituents into the extracellular space (Berghe et al., 2014).

Both caspase-independent mechanisms of cell death and those involving activation of caspase cascades are reported depending on the cellular context (Ryter et al., 2007). In particular, it has been suggested that apoptotic or necrotic cell death can occur depending on the time of exposure and/or the concentration of the oxidative insult (Englert and Shacter, 2002). For example, exposure of PC12 cells to 500 μ M of H₂O₂ resulted in the necrotic death of 90% of the cells 2 hours after the oxidative injury, followed by increased cell death exhibiting features of apoptosis, though with absent caspase-3 activity, after 12–24 hours (Cole and Perez - Polo, 2002). It seems that the appearance of apoptotic markers and CC3 in particular is transient and may only be

detectable within a limited window of time. In my experiments the human hippocampal progenitor cells were treated with *t*-BHP for 1 hour with subsequent 23 hours of a recovery period and then cells were fixed with PFA. Hence, only a snapshot of a certain time point became available for further analysis, which perhaps was not representative for depicting CC3 expression. Indeed, apoptotic cell death can be switched to necrosis during OS by inactivation of caspases due to oxidation of their active site thiol group by oxidants or *S*-nitrosylation. Since caspases contain an active site cysteine which is prone to oxidation, the activity of caspases is optimal under reducing environments. Thus, any deviation from such reducing conditions within an injured cell could be detrimental to caspases and render them inactive (Chandra et al., 2000). In line with this argument, it was demonstrated *in vitro* that H₂O₂ suppresses both the activation and activity of caspases, possibly through modulation of the redox status of the cell and the oxidation of cysteine residues in caspases (Hampton and Orrenius, 1997). Another mechanism that could switch apoptosis to necrosis is a decrease in cellular levels of ATP due to a failure of mitochondrial energy production caused by oxidants, leading to energy requirements for apoptosis not being met (Chandra et al., 2000). Generally, after any incubation period in OS conditions, which is variable across studies, *in vitro* apoptotic cells ultimately shut down metabolism, lose membrane integrity and release their cytoplasmic contents into the culture medium, eventually undergoing secondary necrosis. It is plausible that this scenario occurred in the human hippocampal progenitors subjected to peroxide treatment, as oxidatively damaged cells reached a state of secondary necrosis which was captured by immunocytochemistry.

It is also important to note that the mechanism of OS-induced cell death largely depends on a particular type of ROS. For example, in rat neuronal cultures it was

demonstrated that specific ROS could act as initiators or executioners of neuronal death and lead to different molecular cell death mechanisms. In this study superoxide anion or singlet oxygen induced caspase activation, including CC3, nuclear condensation, phosphatidylserine translocation, and a decrease in intracellular calcium levels, indicative of apoptosis in cultured cerebellar granule neurons. Hydrogen peroxide, however, led to a necrosis-like cell death that did not induce caspase activation, phosphatidylserine translocation, or changes in calcium levels (Valencia and Morán, 2004). In a different study, treatment of cerebellar granule neurons with 100 μ M of H₂O₂ for 15 minutes induced an active caspase-independent but calpain-dependent process of neuronal death distinct from apoptosis (Sée and Loeffler, 2001).

To my knowledge no studies on levels of CC3 expression in MDD patients have been carried out to date. Considering clinical, structural and molecular commonalities between MDD and neurodegenerative disorders, it is possible to extrapolate observations on both increased apoptosis and necrosis from studies on neurodegenerative disorders (Louneva et al., 2008) and speculate that a similar increase may exist in depressed individuals. Necrosis has been mostly associated with pathological conditions such as neurodegeneration and ischaemia–reperfusion-induced injury, both accompanied by OS (Berghe et al., 2014). Interestingly, although a sizeable fraction of cells dying *in vivo* in neurodegenerative processes exhibit morphological features of necrosis, the molecular mechanisms of pathological cell loss partially overlap with the biochemical cascades that mediate apoptosis (Vandenabeele et al., 2010). These observations suggest the occurrence of a distinct kind of regulated cell death that resembles features of both apoptosis and necrosis, which is known as necroptosis or “programmed necrosis” (Ashkenazi and Salvesen,

2014). It is important to mention the role of Ca^{2+} , a ubiquitous second messenger that supports brain physiology maintaining neural integrity and is involved in the control of a broad variety of cellular events including cell death. Although cellular Ca^{2+} overload has been conventionally associated with necrosis, several studies have shown that increases of Ca^{2+} occur both at early and late stages of the apoptotic pathway (Kruman et al., 1998; Tombal et al., 1999). Currently, a common view is that while severe Ca^{2+} dysregulation can promote cell death through necrosis, more controlled intracellular Ca^{2+} increases induced by milder insults promote cell death through apoptosis (Pinton et al., 2008). The notion that Ca^{2+} homeostasis plays an essential role in apoptosis came from the observation that proteins of the Bcl-2 family, which regulate apoptosis, are localized in mitochondria and the endoplasmic reticulum, organelles deeply involved in Ca^{2+} handling (Pinton and Rizzuto, 2006). In particular, it was verified that Ca^{2+} is involved in regulating mitochondrial morphology and release of pro-apoptotic proteins. In HeLa cells upon treatment with ceramide (a pro-apoptotic bioactive lipid), Ca^{2+} release from the endoplasmic reticulum and loading into mitochondria was observed. As a consequence, organelle swelling and fragmentation were detected that were paralleled with the release of cytochrome c, a major apoptosis effector. Notably, these changes were prevented by experimental conditions that lowered Ca^{2+} in the endoplasmic reticulum as well as by antiapoptotic Bcl-2 expression that has been previously demonstrated to reduce Ca^{2+} release from the endoplasmic reticulum (Lam et al., 1994; Pinton et al., 2001). Indeed, mitochondria are endowed with multiple Ca^{2+} transport mechanisms by which they take up and release Ca^{2+} across their inner membrane and thus, regulate local and global Ca^{2+} and Ca^{2+} -sensitive cellular mechanisms. During cellular Ca^{2+} overload, mitochondria take up Ca^{2+} , which, in turn, induces opening of permeability transition

pores, disruption of mitochondrial membrane potential, excessive generation of ROS and cell death. In apoptosis signalling, collapse of mitochondrial membrane and cytochrome c release from mitochondria occur followed by activation of caspases, DNA fragmentation, and cell death (Chinopoulos and Adam-Vizi, 2006; Smaili et al., 2000).

Cells undergoing necroptosis rupture and leak their contents into the intercellular space, but unlike in necrosis, permeabilisation of the cell membrane is tightly regulated. Mechanisms and components of the necroptotic pathway are still being uncovered. In recent years it was described that multiple signal transducers and metabolic processes that can initiate or mediate cellular demolition by necroptosis are ROS generated by mitochondria, suggesting the involvement of redox regulation in this type of cell death. Indeed, excessive generation of ROS has been associated with apoptosis and yet it also occurs during regulated necrosis (Galluzzi et al., 2012). Furthermore, an oxidant-induced cell death known as oxytosis, with morphological characteristics of necrosis but which appears to be regulated by its own signalling pathways that are independent from classical apoptotic pathways (Tan, 2001) was described before “programmed necrosis” was first introduced (Chan et al., 2003). Thus, it is probable that oxytosis is a particular kind of necroptotic cell death, which may occur in neurodegeneration and could also occur in depression.

Together, observations suggest that OS-induced cell death presents a continuum of molecular processes entailing tightly genetically regulated events and mechanistic responses dictated by the redox cellular environment. OS, perturbed energy metabolism, and alterations of disease-related proteins result in Ca^{2+} dependent synaptic dysfunction, impaired plasticity, and neuronal demise. Further research on the exact molecular mechanisms of OS-induced cell death and its potential prevention

may provide a new strategy for the development of cytoprotective agents for neurodegenerative disorders and MDD in particular.

4.3 Insights into changes in neurogenesis upon redox shifts and underlying mechanisms

In this thesis I investigated how neurogenesis is affected in human hippocampal progenitor cells upon induction of redox shifts by *t*-BHP. I then identified potential signalling pathways through which the effects of the oxidant are mediated.

Changes in adult neurogenesis in individuals with neurodegenerative diseases, and depressed patients in particular, is one of the most intriguing matters of current research because it offers tremendous potential for the development of novel therapeutics. The discovery of the existence of adult human neurogenesis and its involvement in the pathogenesis of MDD is a breakthrough which has not yet been pharmacologically addressed, mainly because its functions and most underlying molecular mechanisms are still not fully elucidated. Both alterations in AHN and increased OS have been implicated in depression, however how shifts in redox homeostasis influence human hippocampal progenitors fate remains unclear. The intracellular redox state appears to be a central molecular regulator of the balance between self-renewal and differentiation of neural stem cells and relatively small changes in intracellular redox homeostasis are associated with profound differences in the cell fate (Prozorovski et al., 2015). A key study detected high ROS levels throughout the rat brain during embryonic and early postnatal development. Remarkably, at postnatal stages, cells with the highest ROS were found in the

glomerular layer of the olfactory bulb and in the dentate gyrus of the hippocampus, both areas of known adult neurogenesis, and high ROS cells remained in these structures in the adult rats (Tsatmali et al., 2005). Opposite to a conventional view that excessive ROS can be detrimental to neurons, and that stem cells generally maintain low levels of ROS as a protection against oxidative toxicity, these observations suggest that the generation of ROS may accompany adult neurogenesis. Interestingly, proliferation of stem cells has been associated with a more reducing environment (Halliwell and Gutteridge, 2015), while the redox status of differentiating cells tends to be more oxidized (Lewerenz et al., 2013). Differentiation is a highly energy demanding process and mitochondria are more active during this phase, and as a result more ROS are generated; it is also likely that ROS production at this stage is necessary for facilitating cellular maturation (Lewerenz et al., 2013). Available data provides strong reasons to believe that redox modulation functions as a central integrator of multiple processes related to self-renewal and differentiation, rather than as a controller of a single unique downstream effector pathway. Many different signalling pathways appear to converge on regulation of the redox state, and redox alterations can in turn modulate several different pathways of possible relevance in modulation of self-renewal and differentiation.

Mine is the first study, to my knowledge, that examined the effects of redox shifts in human hippocampal progenitors on neurogenesis and proposed potential mechanisms involved in the underlying changes observed.

4.3.1 Proliferation of the human hippocampal progenitor cells upon redox shifts in the system

Treatment of cells with 25 μ M and 50 μ M of *t*-BHP caused a significant reduction in proliferation, with no difference at 1 μ M and 10 μ M as measured by Ki67 immunolabeling. My observations are in good agreement with studies that provided evidence for a more reduced environment favouring cellular proliferation, however results are conflicting, as some studies reported increased cellular proliferation in response to mild OS insults (Le Belle et al., 2011; Pérez Estrada et al., 2014; Smith et al., 2000; Walton et al., 2012). It was demonstrated that treatment of dividing rat oligodendrocyte progenitor cells with 50nM of *t*-BHP was associated with diminished progenitor division, with one-half of the founder cells differentiated without dividing and the rest undergoing one to three divisions. In this study a reduced environment induced in these cells by 1mM of NAC led to enhanced self-renewal (Smith et al., 2000). In contrast, another study showed increase proliferation of rat NPCs exposed to 100 μ M in H₂O₂ compared to controls (Pérez Estrada et al., 2014). The effects of a redox imbalance on cellular proliferation is likely to depend on the cell type as well as on experimental conditions.

An enhanced proliferation in response to mild OS is usually interpreted as activation of compensatory mechanisms. The cell line HPC0A07/03C utilised in this thesis is a conditionally immortalised line transduced with the c-myc-ER gene that strongly promotes cell proliferation in the presence of 4-hydroxy-tamoxifen (4-OHT) (Dang et al., 1999) and directly regulates a variety of genes, including those that enhance glycolytic activity and oxidative phosphorylation, leading to increased energy production and increased levels of ROS (Dang, 2012; Vafa et al., 2002). Therefore, it is possible that in my experiments the redox-sensitive signalling pathways involved in

promotion of proliferation have reached a plateau, due to an already high proliferation rate and ROS levels induced by c-myc. Thus, shifting the redox status in the system towards more oxidised conditions (1 μ M and 10 μ M of *t*-BHP) did not cause an increased proliferation, whereas higher levels of OS (25 μ M and 50 μ M of *t*-BHP) halted cell proliferation.

Another potential reason for not seeing increased proliferation in my model is the level of activation of NF- κ B, which showed an increase at 10 μ M of *t*-BHP after 24 hours. Both a decrease in the number of proliferating cells in the subgranular zone of the dentate gyrus of the hippocampus and depressive-like behaviour observed in a rat model of induced stress were completely abrogated when rats were pre-administered NF- κ B inhibitors (JSH-23 and SC-514) before the stress (Koo et al., 2010). It is possible that activation of this transcription factor contributed to decreased proliferation of the hippocampal progenitors.

The data obtained in my experiments on decreased proliferation of the human hippocampal progenitors by introducing a more oxidised environment may represent a potential link between the observations on decreased neurogenesis and increased OS observed in MDD.

4.3.2 Differentiation of the human hippocampal progenitor cells upon redox shifts in the system

In the present study human hippocampal progenitor cells exhibited an increased number of neuroblasts (+38% and +30%) and mature neurons (+34% and +33%) upon treatment with 1 μ M and 10 μ M of *t*-BHP, as measured by immunolabeling them with Dcx and MAP2 markers, respectively. It is important to note that the human

hippocampal progenitor cells differentiate in the absence of 4-OHT, meaning that the c-myc gene is not active, and its potential effect to increase ROS levels is therefore absent during this phase. My results support and augment the findings on enhanced neural differentiation induced by endogenous and exogenous pro-oxidant stimulus *in vitro* and *in vivo* (Le Belle et al., 2011; Pérez Estrada et al., 2014; Smith et al., 2000; Tsatmali et al., 2006). For example, when rat oligodendrocytes progenitor cells were exposed to 50nM of *t*-BHP for 5 days during differentiation, 83% of clones consisted wholly of oligodendrocytes, whereas in basal division conditions they reached only 35% (Smith et al., 2000). In a different study, rat NPCs were treated with 100μM of H₂O₂ for 24h and then cells differentiated without any treatment for the next 7 days. As a result, cells exposed to peroxide generated significantly more neurons compared to unexposed controls (Pérez Estrada et al., 2014), suggesting that ROS can trigger differentiation without being present during the entire differentiation phase. Furthermore, murine primary brain-derived neural progenitors treated with H₂O₂ during sphere formation produced significantly higher numbers of neurons as a percentage of total cells than when differentiated in the presence of standard media (Le Belle et al., 2011). Another study that specifically examined the level of ROS in proliferating and differentiating brain cells demonstrated that isolated rat cells that exhibited the lowest reactivity with the ROS-sensing dye were proliferative progenitor cells. When these cells were plated and differentiation was induced, low levels of ROS were detected at day 0 and 2, but after 7 days, fluorescence, indicative of ROS presence, filled the entire cell body and processes in neurons, oligodendrocytes and astrocytes, as detected by immunolabeling (Tsatmali et al., 2006).

In line with my findings these observations provide evidence for physiological roles of ROS and their influence on the neuronal development, however molecular mechanisms were not fully elucidated in these studies. In this thesis I investigated particular signalling pathways that are potentially involved in redox regulation of neurogenesis which is discussed below in **4.3.3**.

Separate attention deserve the *in vivo* studies modelling brain ischaemia–reperfusion-induced injury - a complex neuropathological condition arising after restoration of blood flow to the ischemic tissue accompanied by well-established increased levels of OS (Sanderson et al., 2013). Reperfusion injury is a potentially damaging condition for the brain, and ischemic insults have been reported to induce the formation of new neurons and increased differentiation into mature neurons in humans (Jin et al., 2006), nonhuman primates (Tonchev, 2011) and in the adult rodent brains (Kernie and Parent, 2010; Ruan et al., 2014). Of relevance to MDD, an animal study that investigated the effects of a combination of reperfusion injury with chronic mild stress (commonly used model for inducing depressive-like behaviour in rodents) on adult hippocampal neurogenesis revealed that ischemic rats exhibited increased levels of both new born and differentiated mature neurons in comparison to the control group. Interestingly, in the group with two stressors the number of new born and differentiated neurons was significantly lower than in the ischemic group. Also, the group subjected to reperfusion injury and chronic mild stress demonstrated depressive-like behaviour, with was alleviated by the SSRI citalopram, together with a decrease in neurogenesis (Wang et al., 2008). One can speculate that ischemia-induced neurogenesis could be due to re-connection to the systemic environment through vasculature that intimately surrounds neural stem cells (Filippov et al., 2003)

and provides exposure to an abundance of factors, including ROS that can modulate proliferation, differentiation and survival of the cells (Palmer et al., 2000).

Findings in this thesis provide evidence for ROS acting as secondary messengers and playing an essential role in neurogenesis. However, it is not clear whether ROS-induced neurogenesis is necessarily beneficial, as increased levels of ROS can also lead to various stem cell dysfunctions, which can manifest in different forms such as failure to self-renew, aberrant differentiation or premature senescence and increased apoptosis (Jones and Rando, 2011). The maintenance of balance between self-renewal and differentiation is pivotal for neural stem cells function in development, tissue repair, and homeostasis. In particular, if too many cells are pushed towards differentiation this might lead to a gradual depletion of the stem cell pool. Another possibility is aberrant differentiation, when an abnormally skewed distribution of progeny towards one fate is observed. Furthermore, stem cells can accumulate oxidative cellular damage and reach premature senescence. Additionally, it is not clear whether all new born or matured neurons are capable of appropriately integrating in the existing neural network. One *in vitro* study addressed some of the issues above and revealed a number of interesting facts. In particular, rat brain cells isolated from neurogenic niches with high levels of ROS did not reflect a population that is undergoing the massive cell death or apoptosis that was observed in the cortex under the same conditions. Additionally, using patch clamp analysis it was identified that high ROS-positive cells in neurogenic niches were functional neurons, with neuronal morphologies, normal membrane potentials, and fired repeated action potentials (Tsatmali et al., 2006). These observations provide evidence that increased ROS play a physiological role during neurogenesis, rather than solely being a toxic by-product of an energy-demanding process.

The *in vitro* findings on increased differentiation upon oxidative stimulus obtained in this thesis provide reasons to believe that ROS play a central role in the regulation of neurogenesis. However, the functionality of the neuroblasts and mature neurons differentiated from the human hippocampal progenitor cells upon OS stimulus needs to be further researched *in vitro* and *in vivo* to evaluate the formation of the connections of newly generated cells, stem cell pool renewal, cellular senescence markers and capability of these cells to contribute to cognitive domains. Presumably, ROS facilitated relevant signal transduction pathways which led to an increased differentiation and potential signalling pathways were further investigated in the present study and described below.

4.3.3 MAPK modulation in the human hippocampal progenitor cells by *t*-BHP

I investigated the activation by oxidative stimulus of three main MAPKs that exist in mammalian species: ERK1/2, p38 and JNK. In my experiments no significant increase was observed in the level of phosphorylated MAPKs in the hippocampal cells treated with 1 μ M, 10 μ M and 25 μ M of *t*-BHP during the proliferation and differentiation assay (10 days in total), although a trend towards increase was observed for pERK1/2 at 1 μ M. In order to examine a possible time-dependant activation of the kinases, I evaluated their phosphorylation upon treatment of cells with 1 μ M and 10 μ M of *t*-BHP for 24 hours during proliferation. Again, no significant increase was observed in the level of phosphorylated MAPKs, however a similar trend towards increase was observed for pERK1/2 at 1 μ M of *t*-BHP.

Alterations in redox homeostasis have been reported to induce various signal transduction pathways, thus affecting cell division and/or differentiation. The MAPK

signalling cascades are one of the most ancient and evolutionarily conserved signalling pathways that transduce signal from the cell surface to the nucleus and play essential regulatory roles including cellular growth, differentiation, immune responses and cell death (Zhang and Dong, 2005). MAPKs can be activated by a wide variety of different stimuli, but in general, ERK1/2 is preferentially activated in response to growth factors, and p38 and JNK MAPKs are more responsive to stress stimuli. Interestingly, all these responses are redox-regulated and studies have demonstrated that ROS can induce or mediate the activation of the MAPK pathways along with a number of cellular stimuli that induce ROS production, whereas antioxidants applied after the oxidative insult block MAPK activation (Haddad, 2002; Son et al., 2013). Redox regulation of MAPKs is context specific and may be both upstream and downstream of ROS (Morgan and Kim, 2014). The mechanisms by which ROS can activate the MAPK pathways are not well defined, however plausible mechanisms include oxidation of redox-sensitive proteins in the MAPKs cascades and by directly activating them; another possibility is inhibiting MAPK phosphatases that remove a phosphate group from MAPKs, also resulting in activation of kinase pathways (Kamata et al., 2005).

One of the explanations for not seeing differences in the levels of p38 and pJNK in my experiments across treatment conditions could be due to the fact that immune response is also necessary for triggering these pathways. Although ROS have been shown to be involved in p38 and pJNK initiation, these MAPKs cascades are primarily activated by cytokines such as TNF- α and IL-1 β . As mentioned, no cytokines were detected in my cell model upon *t*-BHP treatment. Another possibility is that the level of oxidative stimulus inflicted to the cells was not sufficient to activate p38 and JNK MAPKs that are known to be mobilized in the stress conditions

for the cells. Furthermore, one of the main signalling pathways that intersects with JNK with regard to ROS and cellular stress is the extensive crosstalk that occurs in multiple ways between JNK and NF- κ B. NF- κ B activation, which was detected in my experiments, is known to prevent sustained JNK activation and thus prevent cell death through both apoptosis and necrosis (Morgan and Liu, 2011a). Therefore, considering the low doses of *t*-BHP that were used in these particular experiments, it is plausible that mostly cytoprotective mechanisms were triggered upon OS insult, resulting in inhibition of activation of stress kinases pathways.

Due to the expenses of the assay, I did not perform more repeats at different doses or time points, and proceeded to evaluate neurogenesis, where I blocked the activation of pERK1/2 during proliferation and differentiation, as discussed in details below.

4.3.4 ERK1/2 as a potential signalling pathway of *t*-BHP-induced neural maturation of the human hippocampal progenitor cells.

In my experiments a trend towards an increase of pERK1/2 was observed when cells were challenged with oxidative stimulus during proliferation only or during both the proliferation and differentiation phases. My findings are consistent with results from other *in vitro* studies, showing ERK1/2 activation upon oxidative stimulus in both undifferentiated and differentiated cells (Ruffels et al., 2004). Interestingly, increased RNA and protein levels of MAPK phosphatase 1 (MKP1) (an enzyme that deactivates ERK1/2) in the postmortem samples of the hippocampus of human subjects diagnosed with depression was found by array-based transcriptome profiling. Furthermore, increased hippocampal MKP-1 expression as a result of stress was observed in the same study, in rat and mouse models of depression. Conversely, chronic

antidepressant treatment normalized both the stress-induced MKP-1 expression and behaviour, whereas mice lacking MKP-1 were resilient to stress (Duric et al., 2010). These observations suggest that a certain level of phosphorylated ERK1/2 is necessary for emotional regulation and my findings show that ERK1/2 can be activated by ROS. The ERK cascade is a major signalling pathway involved in neuronal plasticity, function and survival and thus I also investigated if neural development of the hippocampal progenitors induced by oxidative stimulus is mediated through activation of this pathway. I blocked ERK1/2 activation with the FR180204 inhibitor. My results indicate that the *t*-BHP-induced increase in MAP2-positive neurons was abrogated by blocking this kinase upon redox shifts in the system. However, no effects were observed for Dcx-positive neuroblasts. Thus, these findings indicate that in the human hippocampal progenitor cells ERK pathway is involved in redox-induced neuronal maturation, whereas intermediate process of differentiation into neuroblasts is facilitated through different mechanisms.

ERK phosphorylation is known to be involved in mood regulation, and the majority of *in vivo* studies demonstrate induction of depressive-like behaviour in animals upon inhibition of the ERK pathway (Duman et al., 2007; Réus et al., 2014; Wefers et al., 2012). The mechanisms by which ROS activate the ERK cascade are unclear and the molecular targets are unknown. We know that the ERK pathway is activated by growth factors, such as epidermal growth factor (EGF) which is involved in the regulation of cell proliferation, survival, migration and differentiation, however it was also shown to be directly triggered by ROS (Son et al., 2013). Interestingly, it was suggested that ROS-mediated ERK activation may be an upstream event of the growth factors receptors, that have cysteine-rich motifs. Surprisingly, ROS were shown to affect epidermal growth factor receptor (EGFR) signalling by inhibition of

the receptor internalization, thus increasing the receptor's signalling potential (Verbon et al., 2012). Furthermore, ROS can also directly activate the EGFR in the absence of growth factor receptor ligands (León-Buitimea et al., 2012), which is now referred to as “receptor transactivation” (Son et al., 2013). In my experiments the trend for *t*-BHP-mediated increase in pERK1/2 was observed during both the proliferation and differentiation phases. It is plausible that in the human hippocampal progenitor cells ROS increased the receptor's signalling potential during proliferation, whereas during the differentiation phase, in the absence of EGF, ROS activated EGFR, which resulted in the initiation of the ERK cascade.

Another possible molecular mechanism by which the activation of ERK cascade results in increased formation of mature neurons is through an increase in levels of the neurotrophic factor BDNF. BDNF is one of the downstream molecules of ERK pathway possessing pro-neurogenic, pro-survival and neuroprotective properties, and it is frequently reported to be downregulated in depressed patients (Molendijk et al., 2014). Interestingly, mice with a heterozygous deletion of the BDNF gene showed a depressive phenotype when challenged with a low dose of ERK inhibitor or with a stressor. However, neither the gene deletion nor the low-level inhibitors alone resulted in observable behavioural changes, suggesting a role for intracellular signalling through ERK pathway in the regulation of behaviour in mouse models of depression (Duman et al., 2007). Remarkably, antidepressant treatment (Hunsberger et al., 2009) and non-pharmacological treatments, such as exercise and electroconvulsive therapy (Ruan et al., 2014) have been shown to upregulate the MAPK/ERK signalling pathway and promote neurogenesis in animal models of depression. Likewise, it was demonstrated in cultured cortical neurons that neuroprotective effects of BDNF rely on ERK activation (Sun et al., 2008). Considering these observations, it would be

instructive to measure the levels of BDNF in the differentiated human hippocampal progenitor cells and identify whether the redox-induced neurogenesis that is mediated through ERK pathway is due to an increase of this neurotrophic factor. It would also be of interest to measure other redox signalling pathways that have been shown to influence neurogenesis, such as the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/Akt) pathway. In combination with exogenous ROS stimulation, pharmacological inhibition of PI3K eliminated the increased differentiation of murine and human neural progenitors into mature neurons caused by H₂O₂ (Le Belle et al., 2011). It is possible that a redox induced PI3k/Akt pathway contributed to the increased neuroblast formation in my experiments.

To conclude, redox regulation of neurogenesis is a tightly balanced complex process, which mainly depends on the cellular context. Further research is needed to identify potential strategies for targeting redox-mediated signalling pathways that are involved in neurogenesis modulation.

4.4 Crosstalk between redox and inflammatory systems

4.4.1 Activation of Nrf2 and NF-κB transcription factors with no signs of inflammation

In my model I investigated whether the oxidative stimulus leads to activation of two transcription factors: NF-κB, which is a master regulator of immune response, and Nrf2, the major antioxidant defense transcription factor. Additionally, I examined if the redox-induced upregulation of NF-κB modulates the production of cytokines in the human hippocampal progenitor cells, considering that increased levels are

constantly detected in MDD patients as evidenced by several meta-analyses (Dowlati et al., 2010; Howren et al., 2009; Valkanova et al., 2013). In my experiments *t*-BHP treatment of the human hippocampal progenitor cells caused activation of both Nrf2 and NF- κ B transcription factors in a dose-dependent manner, however no signs of elevated cytokines across all conditions treated with a range of peroxide concentrations were detected.

A significant increase in the amount of nuclear Nrf2 was observed in the hippocampal progenitors at 50 μ M (+46%) and at 100 μ M (+113%) of *t*-BHP after 1 hour. Induction of Nrf2 upon OS is a common finding (Dell'Orco et al., 2016; Haskew-Layton et al., 2010). Interestingly, it was demonstrated *in vitro* that low and moderate doses (125 μ M, 250 μ M and 500 μ M) of hydrogen peroxide increased Nrf2 activation, whereas higher doses (1mM and 2mM) decreased its transcription activity (Ning et al., 2010). In my experiments I checked Nrf2 activation only when using doses of *t*-BHP up to 100 μ M, because higher doses led to a significant decrease in cell viability, when antioxidative mechanisms were not capable of resisting the oxidative damage.

More and more evidence suggests that Nrf2 contributes to the pathogenesis of MDD. A recent study reported that mice with a depression-like phenotype induced by repeated social defeat stress exhibited lower expression of Keap1 and Nrf2 proteins in the prefrontal cortex, CA3 and dentate gyrus of hippocampus, whereas pre-treatment of the animals with the Nrf2 activator sulforaphane prevented the depression-like behaviour (Yao et al., 2016). Importantly, the role of Nrf2 in mood regulation extends beyond its permutation of antioxidative defence system. It was demonstrated that Nrf2 deletion results in depressive-like behaviour in mice and also in altered levels of neurotransmitters, crucial for emotional processing (Martín-de-Saavedra et al., 2013; Wojnicz et al., 2016).

Furthermore, in addition to regulation of the antioxidant defence by Nrf2, recent evidence suggests crucial involvement of Nrf2 in the modulation of the inflammatory system (Ma, 2013). In particular, the immunosuppressive properties of Nrf2 were studied in human immature dendritic cells, where in the presence of low concentrations of the Nrf2 inducer inorganic arsenic, the ability of the cells to release key cytokines in response to different stimulating agents such as LPS was repressed (Macocho et al., 2013). Similarly, higher serum levels of proinflammatory cytokines were reported in Nrf2 knock-out mice than in wild-type mice (Yao et al., 2016). Contrary to the widely accepted view that Nrf2 suppresses inflammation through redox control, a recent study demonstrated that Nrf2 opposes the transcriptional upregulation of proinflammatory cytokine genes (Kobayashi et al., 2016). Both a microarray and Nrf2 chromatin immunoprecipitation (ChIP)-seq analyses revealed that Nrf2 binds to the proximity of proinflammatory cytokine genes, including IL-6 and IL-1 β , and inhibits their expression induced by LPS. Furthermore, the Nrf2-mediated inhibition of proinflammatory cytokine genes was independent of ROS elimination, as NAC treatment alone without Nrf2 induction did not affect cytokine mRNA expression. Further *in vivo* imaging analysis revealed that Nrf2 activation inhibits IL-6 induction in a murine inflammation model and alleviates inflammatory phenotypes (Kobayashi et al., 2016).

NF- κ B is another critical transcription factor involved in the pathophysiology of MDD that has been reported to be both down- or up-regulated in OS conditions (Oliveira-Marques et al., 2009). In my experiments treatment of the cells with *t*-BHP for 1 hour caused a significant increase in NF- κ B activation at 50 μ M (16%) and 100 μ M (17%), the same concentrations that caused Nrf2 activation. The crosstalk mechanism between Nrf2 and NF- κ B is complex and is still under elucidation

(Buelna-Chontal and Zazueta, 2013). Treatment of the cells with *t*-BHP for 24 hours caused a much smaller but significant increase in NF- κ B activation at 10 μ M (4%) with no effect at 1 μ M. Since higher concentrations of *t*-BHP are toxic when applied for 24 hours, they were not tested in this experiment. Thus, these data suggest that redox NF- κ B activation occurs at different levels and duration of oxidative stimulation.

Opposite to my hypothesis that redox activation of NF- κ B would lead to increased production of cytokines in the human hippocampal cells, no inflammatory proteins were detected in the cellular supernatant as measured by a 25-plex panel. There is a number of reasons for these observations. First of all, although NF- κ B activation is mainly associated with promulgation of pro-inflammatory cascades, it is also known to regulate transcription of a variety of genes differentially affecting proliferation, maturation, and survival hugely depending on cell type, localization, conditions and stimulus applied (Koo et al., 2010; Morgan and Liu, 2011b). Once NF- κ B is triggered, it is not clear to what extent it needs to be activated in terms of duration and degree of the effect to initiate an inflammatory response. Therefore, there is a possibility that in my experiments the activation of NF- κ B was a transit event in response to the acute oxidative stimulus that did not lead to an immune reaction. Finally, given the immunosuppressive capacity of Nrf2, it is possible that in the human hippocampal progenitor cells challenged with OS, Nrf2 inhibited the production of cytokines via clearing up ROS and also by directly blocking gene transcription.

While both increased OS and inflammatory biomarkers are frequently reported in depressed patients, it is still not clear what the initial event that triggers the downstream cascades is, and complex interactions between redox and inflammatory

systems are not fully elucidated. It has been hypothesised that OS is a key determinant of neuroinflammatory response, potentially critical to emotional wellbeing, as enhanced inflammation also leads to an increased ROS production (Salim, 2016). Considering that MDD has been associated with increased OS and activation of inflammatory pathways, studies that identify Nrf2 as the upstream regulator of antioxidant defences and cytokine production provide a molecular basis for an Nrf2-mediated antioxidative and anti-inflammatory approach.

4.4.2 Kynurenine pathway

In this thesis I investigated if the oxidative challenge of the human hippocampal cells leads to perturbations in the kynurenine pathway. Quinolinic acid (QUINA), 3-hydroxyanthranilic acid (3-HANA), kynurenic acid (KYNA) and 3-hydroxykynurenine (3-HK) were not detected in neither the supernatants collected from cells treated with 200µM, 500µM and 1000µM of *t*-BHP after one hour, or after subsequent 23 hours. Kynurenine (KYN), tryptophan (TRP) and anthranilic acid (ANA), were detected in all samples, however no significant differences across conditions were observed.

Alterations in the kynurenine pathway have been implicated in the pathogenesis of depression, because catabolism via kynurenine pathway may divert tryptophan from the serotonin synthetic route, critical for emotional well-being (Maes et al., 2011b). The kynurenine pathway includes metabolism of KYN to either the potentially neuroprotective KYNA, or to the excitotoxic free radical generator compounds 3-HANA and QUINA. It was proposed that an imbalance between QUINA and KYNA resulting in increased neurotoxicity is key in mood disorders (Maes et al., 2011a; Savitz et al., 2015). Increased levels of 3-HANA and QUINA, together with IDO, have been associated with MDD (Gabbay et al.,

2010; Young et al., 2016). Nor KYNA, neither 3-HANA or QUINA have been detected in my samples, whilst ANA was present in all samples, suggesting that in my experiments kynurenine pathway went towards ANA, which was reported to have both pro-oxidant and scavenging as well as anti-inflammatory properties upon interaction with copper (Gaubert et al., 2000; Ocampo et al., 2014).

Importantly, the kynurenine pathway is known to be affected by inflammation and OS (Tan et al., 2012; Lovelace et al., 2016). The pathway converts the serotonin precursor tryptophan to kynurenine, catalysed by indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO), potentially leading to less serotonin, a principal neurotransmitter in mood regulation (Myint et al., 2007). It has been identified that shifting the tryptophan metabolism towards kynurenine is mainly due to increased IDO activity that is triggered by pro-inflammatory cytokines such as IFN- γ , TNF- α , IL-2 and IL-1 β (Zunszain et al., 2012a), and also directly by ROS (M Maes et al., 2011b). Interestingly, tryptophan and kynurenine have been reported to possess scavenging properties and can be considered as potential antioxidants (Ocampo et al., 2014). Therefore, another possibility is that the tryptophan and kynurenine present in my samples counteracted the effect of *t*-BHP on the pathway activation.

Both neuroprotective and neurotoxic properties of metabolites of the kynurenine pathway have been investigated in regards to the pathogenesis of MDD. Due to the dual effects of these metabolites and the high degree of modulation of the pathway by inflammatory cytokines, metals, pH and the redox environment, it is challenging to establish a precise mechanism by which cellular alterations can be produced. In particular, as OS and inflammation are intimately linked with each other in the pathophysiology of depression, studying the effects of both together, as happens *in vivo*, would be of interest.

4.5 Investigating the antioxidative properties of antidepressants and omega-3 fatty acids

4.5.1 NAC and GSH, but not antidepressants exhibited antioxidative properties

In this thesis I tested sertraline, venlafaxine and ketamine for their antioxidative properties in the human hippocampal progenitor cells treated with the doses of *t*-BHP that significantly reduced cell viability. I also validated the model by testing the antioxidants NAC and GSH, both of which exerted antioxidative characteristics and prevented the decrease in cell viability inflicted by the oxidative damage.

Pre-treatment of the cells with 1mM or 10mM of NAC did not have a significant preventing effect on cell viability when compared to the cells treated with 100μM and 500μM of *t*-BHP only. However, pre-treatment of the cells for 1 hour followed by co-treatment with a higher dose of NAC, 100mM, and either 100μM or 500μM of *t*-BHP caused a reduction in cell viability to 85% and 76% respectively, being significantly different from the conditions where cells were treated with peroxide only, when cell viability was reduced to 52% at 100μM and 37% at 500μM. In this experimental paradigm sertraline, venlafaxine and ketamine did not prevent the decrease in cell viability caused by *t*-BHP.

Following these results, I decided to increase the pre-treatment duration with all compounds under investigation to 24 hours and also to use cell permeable GSH instead of NAC, hypothesizing that GSH might be antioxidative at lower doses than its precursor. Cells were pre-treated for 24 hours with 1mM and 10mM of GSH, followed by 1 hour co-treatment with either 100μM or 500μM of *t*-BHP. In this situation 1mM and 10mM of GSH plus 100μM of *t*-BHP caused reductions in cell viability to 98% and 91% which presented a significant difference when compared to

the cells treated with 100 μ M of *t*-BHP only, reaching 75%. In the same experiment 1mM and 10mM of GSH co-treated with 500 μ M of *t*-BHP caused changes in cell viability to 76% and 73%, which was also significantly different when compared to the cells treated with 500 μ M of *t*-BHP only reaching 46%. Sertraline, venlafaxine and ketamine again did not demonstrate the ability to prevent the decrease in cell viability caused by oxidative damage.

The decision on testing antidepressants for their antioxidative properties in my model was prompted by numerous reports in the literature on the ability of these compounds to reduce OS in depressed patients or in animal models of depression. The molecular mechanisms of their antioxidant effects remain elusive, and I wished to generate insights on this phenomenon. To my knowledge, there are no previous studies investigating the antioxidative effects of sertraline, venlafaxine and ketamine in human brain cells. My *in vitro* model is especially useful for addressing these molecular mechanisms, because it eliminates a huge number of confounding factors present in *in vivo* models. A likely explanation of my negative findings on the ability of antidepressants to prevent the reduction in cell viability inflicted by the oxidative damage is that sertraline, venlafaxine and ketamine do not possess direct scavenging characteristics or affect pathways that are primarily involved in the antioxidant defence. Nevertheless, these antidepressants have been demonstrated to positively modulate OS biomarkers *in vivo*, and it is most probable that their effect is mediated through indirect mechanisms influencing other factors, such as the immune response. For example, venlafaxine was shown to reduce NF- κ B and IL-6 protein secretion induced by IL-1 β in the human hippocampal progenitor cells (Horowitz et al., 2015) utilised in this thesis. Possibly, antioxidative effect of venlafaxine *in vivo* could be

mediated through downregulation of NF- κ B and proinflammatory cytokines that in turn determine the oxidative state.

4.5.2 EPA, but not DHA, protected cells from oxidative damage

Among all the compounds I investigated implementing 24 hours of pre-treatment (sertraline, venlafaxine, ketamine, EPA and DHA) only EPA exhibited antioxidative properties, by completely preventing the reduction in hippocampal cell viability caused by 100 μ M of *t*-BHP. No effect of EPA on viability of cells treated with 500 μ M of *t*-BHP was observed, most likely because the oxidative damage was too severe.

My findings are consistent with a number of *in vivo* and *in vitro* studies that have demonstrated antioxidant effects of omega-3 fatty acids (Giordano and Visioli, 2014). Positive effects of omega-3 fatty acids on systemic OS have been shown in various pathological conditions, including depression in humans and animals (Castillo et al., 2014; Guermouche et al., 2014). However, there is widespread concern that intakes of long chain omega-3 fatty acids might increase circulating concentrations of lipoperoxides, potentially interfering with the immune system. Indeed, the oxidizability of the omega-3 fatty acids and DHA and EPA in particular (at least *in vitro*) is very high, due to their high number of double bonds (Halliwell and Gutteridge, 2015). Nevertheless, no evidence was found for pro-oxidant effects of omega-3 fatty acids on lipid peroxidation by a comprehensive review that summarised *in vivo* findings on the levels of F2-isoprostanes in patients receiving fish oil supplements (Mori, 2013). Consequently, it has been suggested that high doses of

omega-3 fatty acids might trigger OS, whereas lower doses could exert antioxidant activities (Guillot et al., 2009).

A number of *in vivo* and *in vitro* studies that have demonstrated antioxidant effects of PUFAs proposed molecular mechanisms underlying its antioxidant activities, such as Nrf2 activation and NADPH oxidases inhibition (Giordano and Visioli, 2014; Kusunoki et al., 2013; Liu et al., 2014; Richard et al., 2008; Zhang et al., 2014). Contrary to my expectations based on reported antioxidative properties of DHA *in vitro*, in my model it did not prevent the oxidative cell damage. In particular, DHA activated Nrf2 and its downstream genes, protecting mouse neural progenitor cells from the oxidative injury induced by H₂O₂ (Liu et al., 2014). In endothelial cells, DHA was shown to inhibit one of the NADPH oxidases and hence decreased ROS production (Richard et al., 2009). The actions of different compounds hugely depend on their concentration, the experimental paradigm and the cell type. Interestingly, a combination of both DHA and EPA showed neuroprotective effects in mice neurons tested in a similar *in vitro* experimental paradigm as the one in this thesis. Following the initial observation that the fatty acids attenuated ischemic neuronal injury in mice via Nrf2 and heme oxygenase-1 (HO-1) activation, an *in vitro* experiment was set up where cortical neurons were pretreated with 10μM, 20μM, 40μM and 100μM of EPA or DHA for 24 hours, and then subjected to oxygen glucose deprivation for 1 hour to mimic *in vitro* ischemia. Cells were returned to normal culture media supplied with the same concentration of DHA or EPA for the following 24 hours. Both DHA and EPA reduced the percentage of dead neurons and the level of lactate dehydrogenase (LDH), an enzyme that is released when cells loose membrane integrity, in a dose-dependent manner between 10μM and 100μM, with the highest pronounced effect at 20μM, indicating a neuroprotective effect (Zhang et al., 2014).

Results suggest an optimal dose for the protective effects, which may not have been the one used for DHA in my experiments. Nevertheless, in my experiments EPA prevented the reduction in cell viability induced by *t*-BHP, which is in line with other *in vitro* data examining the antioxidative properties of this fatty acid (Sakata et al., 2013). Similarly, EPA-enriched phospholipids exhibited neuroprotective effects against H₂O₂ or *t*-BHP-induced cytotoxicity in PC12 cells, as measured by MTS and LDH assays (Wu et al., 2014). Interestingly, it was demonstrated recently that MDD responds to EPA or EPA + DHA treatments, differentially or synergistically, being most effective with higher doses of EPA combined with lower doses of DHA (Song et al., 2016). Also, it was identified that chronic or severe MDD cognitive decline is associated with lower EPA but not DHA concentrations in erythrocyte membranes (Chiu et al., 2012). Moreover, findings coming from animal studies suggest that low levels of EPA compared to DHA in brain phospholipids may be the result of EPA rapid β -oxidation, that occurs quicker than DHA upon uptake by the brain (Chen et al., 2009). Thus, it is plausible that the brain requirement for EPA is higher than for DHA, as EPA gets oxidised more when the level of OS increases in pathological conditions, such as MDD.

My results support the notion of EPA possessing cytoprotective characteristics against oxidative damage, which contributes to the pathophysiology of MDD and it is particularly pertinent in light of recent meta-analyses emphasising the importance of EPA over DHA in the antidepressant efficacy of fish oil preparations (Lin et al., 2010; Martins et al., 2012).

4.6 Modulation of gene expression by EPA and GSH

Following the results where EPA was shown to be cytoprotective against oxidative damage in my model, I wished to investigate some underlying molecular mechanisms of its effect. I hypothesized that the antioxidative properties of EPA could be mediated by antioxidative gene regulation. Thus, using the same experimental design as in assessing the capacity of compounds to prevent the reduction in cell viability induced by *t*-BHP, I tested the effect of *t*-BHP on its own, as well as that of EPA and GSH in the presence of the oxidative insult, on the regulation of the following genes: *NQO1*, *SLC1A1*, *HMOX1*, *SLC7A11*, *GCLM*, *Keap1*. I decided to test EPA and GSH in the presence of oxidative stimulus but not on their own, because I wanted to find out whether these drugs are capable of regulating these genes in the presence of oxidative stimulus, since I am interested if MDD patients who have an increased oxidative status and may benefit from the compounds under investigation. Considering that 500μM of *t*-BHP caused a dramatic decrease in cell viability (46%) and EPA was not effective at this dose of *t*-BHP, I chose to use 100μM of *t*-BHP for all gene expression experiments.

4.6.1 *Keap1* and *HMOX1*

Treatment of the cells with *t*-BHP only, or EPA and GSH in the presence of *t*-BHP, led to downregulation of *Keap1*: *t*-BHP only of -20%, *t*-BHP plus EPA of -35% and *t*-BHP plus GSH of -47%. *Keap1* is the gene that codes for the protein that anchors Nrf2 to cytoplasm and thus keeps it inactivated (**Figure 47**). Downregulation of *Keap1* by *t*-BHP is consistent with my results indicating increased levels of nuclear Nrf2 translocation upon 100μM of *t*-BHP treatment (section 3.4.1). Interestingly,

although EPA and GSH would potentially reduce OS due to their scavenging capacity, these molecules also promote an antioxidant defence by downregulating *Keap1*, which subsequently causes higher levels of Nrf2 being liberated from the cytoplasm and translocated to the nucleus, where it can initiate transcription of the antioxidative genes. It is plausible that both EPA and GSH positively modulate Nrf2 activation by downregulating *Keap1*. In line with data obtained in this thesis, it was shown in brain endothelial cells challenged with hydrogen peroxide that GSH not only prevents cellular oxidative damage, but also increases the basal level of Nrf2 proteins and upregulates Nrf2 protein levels decreased by H₂O₂ treatment (Song et al., 2014). EPA as well as DHA have been previously demonstrated to upregulate the levels of Nrf2 in rats and in rat primary cortical neuron cultures (Zhang et al., 2014). However, to my knowledge, this is the first study showing in human hippocampal progenitor cells the ability of EPA and GSH to downregulate *Keap1*, potentially causing activation of Nrf2.

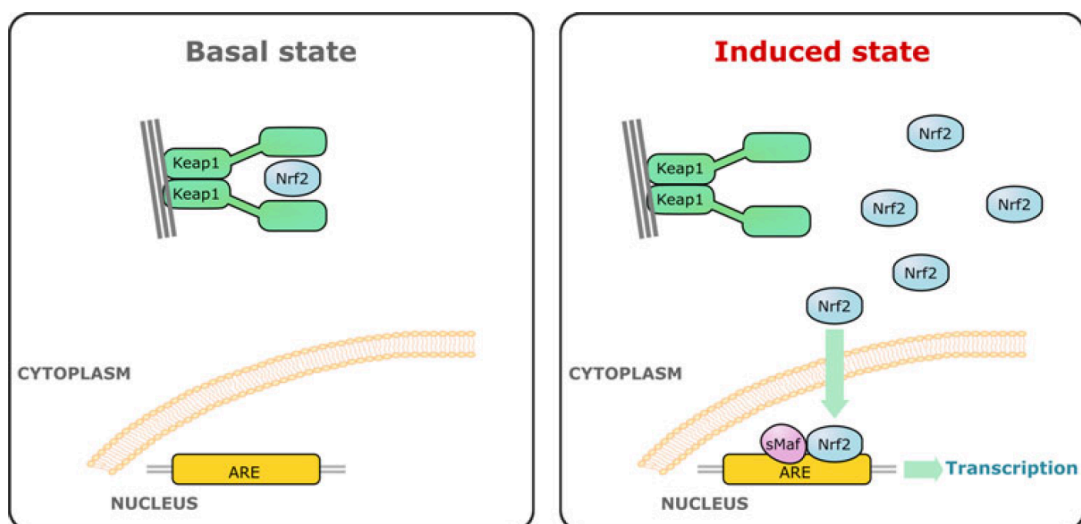


Figure 47. Release model of regulation of the Keap1–Nrf2 pathway. Dimeric Keap1 sequesters Nrf2 in the cytoplasm by binding to the actin cytoskeleton. Inducers react with specific cysteine residues in Keap1, leading to release of Nrf2, allowing the transcription factor to translocate to the nucleus and turn on the expression of cytoprotective genes (Baird and Dinkova-Kostova, 2011).

Treatment of the hippocampal cells with *t*-BHP upregulated *HMOX1* gene by +62% and upon co-treatment with *t*-BHP plus EPA by +92%. *HMOX1* is a downstream Nrf2 gene, which is highly responsive to oxidative stimulus and codes for the enzyme heme oxygenase 1 (HO-1). HO-1 catalyzes the degradation of protein-unbound heme, that accelerates the production of ROS. HO-1 is one of the key players in the oxidative defence mechanism, and plays a fundamental role against the free heme mediated oxidative process (Morimatsu et al., 2012). While in my experiments EPA in the presence of *t*-BHP upregulated *HMOX1* with a stronger signal, surprisingly, no difference for this gene expression was observed with GSH in the presence of *t*-BHP. In this case, since GSH is a strong free radical scavenger, it is possible that its addition to the cells attenuated levels of OS that induced the upregulation of *HMOX1* in other conditions. These results are in line with other studies that addressed the antioxidative mechanisms of fish oils. It was demonstrated in mice and *in vitro* that omega-3 PUFAs diminished ischemic neuronal injury by Nrf2 and HO-1 activation through generation of 4-HNE (Zhang et al., 2014). Furthermore, in adipocytes the supplementation with omega-3 PUFAs also led to activation of Nrf-2 and HO-1 expression (Kusunoki et al., 2013).

4.6.2 *NQO1* and *GCLM*

Data obtained in this thesis indicated no significant difference in the expression of *NQO1* and *GCLM* genes across all experimental conditions. *NQO1* gene codes for the redox-regulated flavoenzyme NADPH:quinone oxidoreductase 1 (NQO1), protecting against OS induced by detoxifying quinone derivatives. NQO1 functions as a two electron donor providing a substrate that competes with the formation of ROS. *GCLM*

gene codes for the glutamate cysteine ligase modifier (GCLM) subunit of the rate-limiting protein of the GSH synthesis glutamate cysteine ligase (GCL). The GCL subunits are often coordinately induced in response to OS, hormones and growth factors, but distinct transcriptional and post-transcriptional mechanisms mediate their differential rates and levels of induction (Franklin et al., 2009). Out of two subunits I chose to measure GCLM, because it was demonstrated to be a limiting enzyme for GCL enzyme formation in most cell types and tissues (Chen et al., 2005; Lee et al., 2006), and therefore an increased expression of GCLM alone is likely to be an effective mechanism for enhancing cellular GCL activity. Importantly, while neither *NQO1* (Pae et al., 2007) nor *GCLM* (Berk et al., 2011) polymorphisms appear to be related to a susceptibility to depression, they appear to be associated with the response to antidepressant drugs (Mendez-David et al., 2015). Both *NQO1* and *GCLM* genes are downstream targets of Nrf2, and have been also reported to be regulated by other transcription factors, including NF- κ B (Lu, 2013). Perhaps in my experiments no induction of *NQO1* and *GCLM* genes has been observed due to regulation of these genes by other than Nrf2 pathways influencing its expression. Furthermore, epigenetic modifications (heritable alterations in gene expression that occur without changes in the primary DNA sequence) have been reported to be profoundly involved in OS responses. ROS can cause serious DNA lesions and lead to mutagenesis. In addition, many Nrf2-activating compounds have been identified as epigenetic modulators, and the expression of several Nrf2-target genes has been found to be regulated epigenetically. Circumstantial evidence suggests that different epigenetic layers may be engaged in complex crosstalk to establish and maintain different chromatin states. Keap1 and Nrf2 expression could be regulated by methylation/demethylation of CpGs in the promoter regions, acetylation/deacetylation and

methylation/demethylation of histones, or targeting of mRNAs by miRNAs. Moreover, the above-mentioned epigenetic modifications may not function alone, but they may be linked to each other and work in combination to regulate gene transcription (Guo et al., 2015). Finally, target genes can be also epigenetically modified. For example, it was demonstrated *in vitro* that hypermethylation of the CpG DNA island of the *NQO1* gene leads to its downregulated transcription (Tada et al., 2005). Another possible explanation of my findings is that the time point (1 hour after OS insult followed by 23 hours of incubation in fresh media) was not representative of upregulation of *NQO1* and *GCLM* expression and an increase in gene expression occurred within 23 hours of incubation in fresh media.

4.6.3 Modulation of system x_c^- and glutamate transport by the compounds under investigation via gene expression

In my experiments *t*-BHP treatment of the human hippocampal progenitor cells caused a downregulation of *SLC7A11* gene of -25%, whereas EPA in the presence of *t*-BHP prevented the downregulation of this gene caused by oxidative injury and upregulated it by +38% when compared with the peroxide treatment. In contrast, GSH did not demonstrate similar effects to EPA and downregulated this gene even further, for -42% from the vehicle condition. Treatment with the peroxide alone, or with EPA in the presence of the oxidative stimulus, did not cause significant changes in *SLC1A1* expression. Surprisingly, GSH plus *t*-BHP led to a downregulation of *SLC1A1* gene of -35% when compared with vehicle.

SLC7A11 gene codes for the determinant xCT subunit of the cystine/glutamate exchange system (system x_c^-), which is the main provider of cysteine for GSH

synthesis. System x_c^- plays a pivotal role in redox homeostasis as well as neuronal signalling by regulating the glutathione production which occurs via the exchange of intracellular glutamate with extracellular cystine at a 1:1 molar ratio. Dysregulation of GSH homeostasis and alterations in glutamate neurotransmission have been vastly implicated in the pathophysiology of MDD (Duffy et al., 2015; Lewerenz and Maher, 2015). It was postulated that any perturbation in the system x_c^- system function may contribute, directly or indirectly, to the pathophysiology of a variety of CNS disorders including depression, making it a promising target for treating CNS disorders (Patel et al., 2015). Therefore, I wished to investigate how *SLC7A11* gene expression is regulated in the human hippocampal cells by oxidative stimulus and potentially modulated by the antioxidative EPA and GSH. Generally, the xCT subunit can be induced by OS in both an Nrf2-dependent or -independent manner. Contrary to my expectations, *SLC7A11* was downregulated by *t*-BHP treatment, presumably due to excessive release of glutamate into the extracellular space as a result of increased cell death inflicted by oxidative damage. Indeed, many *in vitro* studies demonstrated inhibition of system the x_c^- by glutamate concentrations greater than 20 μ M (Kritis et al., 2015). Also, xCT has been recently reported to be regulated by the activating transcription factor 4 (ATF4) and Nrf1 and the effect of *t*-BHP treatment on its activity is unclear (Massie et al., 2015). EPA, however, reversed the oxidative *SLC7A11* downregulation, demonstrating another important determinant of its antioxidative effects.

Interestingly, GSH downregulated *SLC7A11* and the biological rational for this would be a self-regulation of the system via a negative feedback loop. Specifically, exogenously added GSH can inhibit *SLC7A11* in order not to recruit more cystine for more GSH synthesis, since it may be already present in abundance. In line with this

notion GSH downregulated *SLC1A1*, the gene that codes for the EAAT3 transporter, whose main function is to regulate the extracellular glutamate concentration and maintain its concentration at low physiological levels to avoid toxic effects (Krzyżanowska et al., 2014). At excitotoxic concentrations, glutamate triggers the rise of intracellular Ca^{2+} levels, followed by upregulation of ROS production, dysfunction of mitochondria, and release of lysosomal enzymes, eventually leading to neuronal oxytosis (Tan, 2001). As such, glutamate accumulation also inhibits system x_c^- and depletes neurons of glutathione's reducing potential, leading to free radical accumulation (Kritis et al., 2015). Thus, after release into the synaptic cleft in exchange for cystine, glutamate is rapidly removed through EAATs into glial cells and neurons. So, GSH inhibited the system x_c^- and potentially shifted the homeostasis towards less cystine being imported into the cell and concomitant decrease in extracellular glutamate release. These perturbations, in turn, required the adjustment of the rate of the glutamate clearance, which was decreased by downregulation of *SLC1A1*. This idea is supported by *in vivo* research in xCT knock-out mice, which exhibited a strong decrease in extracellular glutamate levels (De Bundel et al., 2011). Interestingly, a recent animal study indicated anxiolytic and antidepressant-like effects in system x_c^- deficient mice, suggesting that under physiological conditions glutamate release via x_c^- system mediates aspects of higher brain function related to mood regulation (Bentea et al., 2015). Data obtained in this thesis provide further evidence for system x_c^- being responsive to changes in redox environment, possibly tightly regulating the balance between GSH synthesis and glutamate release, both implicated in the pathogenesis of MDD.

4.6.4 Summary on gene expression modulation by EPA and GSH

In this thesis I have demonstrated that EPA and GSH exert antioxidative properties in the presence of an oxidative injury via differentially regulating the expression of genes involved in an OS response. In particular, both EPA and GSH amplified the Keap1 gene downregulation that was inhibited by *t*-BHP, potentially leading to an increase in Nrf2 activation. EPA also upregulated *HMOX1* and reversed the *t*-BHP-induced downregulation of *SLC7A11*, suggesting that its antioxidative properties extend beyond being a mechanistic target for oxidation.

Additionally, I provided evidence that GSH regulates the x_c^- system by downregulating *SLC7A11* gene, potentially leading to a reduction of excessive extracellular glutamate, that is involved in almost every aspect of physiological brain functioning, including the control of emotion which has been implicated in the pathogenesis of MDD. Furthermore, GSH downregulated *SLC1A1*, possibly promoting a settling to equilibrium between extracellular glutamate and GSH synthesis *de novo*.

Overall, data obtained in this thesis on the effects of EPA and GSH on gene expression considerably contribute to the existing knowledge on their cytoprotective properties. The present study also emphasises the role of system x_c^- and its redox regulation by GSH, which might constitute the basis of innovative interventions in mood disorders.

4.7 Methodological considerations

The findings obtained in this thesis have a number of possible limitations, regarding the cell and induced OS model that should be considered when interpreting the data.

4.7.1 The use of an *in vitro* system

I have used a conditionally immortalised human embryonic hippocampal progenitor cell line to model exogenously-induced OS and to examine the effects of redox shifts on cell viability, neurogenesis, regulation of transcription factors and downstream molecular cascades, as well as the capability of various antidepressant compounds to prevent the inflicted oxidative damage.

As with all *in vitro* models, the main limitation of the study lies in the challenge to extrapolate findings from cells to the whole organism. Isolated cells in culture are not embedded in their natural environment, therefore such a system necessarily provides a simplistic representation of the whole organism. Hence, data obtained from *in vitro* studies must be interpreted with caution in order not to draw erroneous conclusions about the more complex, entire organism. However, the cells used in the present study represent one of the only means to investigate the processes relevant to the pathogenesis of depression and the effects of antidepressant compounds in human brain cells, and, in particular, hippocampal progenitor cells that are capable of neurogenesis with pertinent relevance to depression. Furthermore, the simplicity of *in vitro* systems also has advantages, as it allows the targeted examination of specific molecular mechanisms such as redox-induced activation of transcription factors, without confounding influences which cannot be eliminated *in vivo*, but simply omitted in a cell culture system.

Previously, studies that have looked at biomarkers of OS in depression and effects of antidepressants, antioxidants and fish oils on the oxidative status were mainly conducted in animal models of depression and in the peripheral samples from humans. *In vitro* studies that have researched similar parameters were performed in cellular models obtained from rodents. These approaches have been instructive, but

there are difficulties translating effects found in rodents (either *in vivo* or *in vitro* models) to humans, due to the possibility of species-specific effects. Findings from peripheral human samples are also difficult to extrapolate to the CNS, given that human neural cells might not be directly similarly affected. In contrast, the present model allows the investigation of molecular mechanisms potentially associated with MDD directly on human neural cells coming from the hippocampus - a brain area known to be affected in depression. What is more, this approach enables the comparison with findings in animal brains and peripheral human samples, providing a complementary set of observations.

Previous findings from this cell model have been replicated in human samples, and conversely, effects thought to occur in depressed subjects have been recapitulated in this cell model, suggesting that the model possesses translational relevance and may be a useful system for generating insights into some molecular mechanisms of depression. For example, our laboratory had identified that antidepressants upregulate the pro-neurogenic gene *p11* (Anacker et al., 2011) and this finding was then replicated in peripheral blood mRNA of depressed patients before and after eight weeks of treatment with antidepressants (Cattaneo et al., 2013). Additionally, it was also demonstrated that IL-1 β (Zunszain et al., 2012a) and dexamethasone (Anacker et al., 2011) (both considered depressogenic insults) decrease neurogenesis in the present cell model. Furthermore, dexamethasone treatment of these cells demonstrate epigenetic and gene expression changes in the stress-regulated gene *FKBP5*, that are identical to those identified in peripheral blood mRNA of patients who have experienced childhood maltreatment, known to be a major risk factor for depression (Klengel et al., 2013).

Thus, this *in vitro* model offers means to investigate molecular processes pertinent to depression that could potentially be translated in an *in vivo* studies with species-specific relevance and without the need for animals. Reduced usage of animals is an ethical imperative in the research community and therefore performing screening of various compounds for specific qualities which often requires a lot of modifications and steps is more appropriate in a cell culture. While it is difficult to be certain how readily translatable the findings in hippocampal progenitor cells are to processes in the human brain, studies continue to emphasise their importance in memory processes and other functions (Kropff et al., 2015).

4.7.2 Cell type

4.7.2.1 c-Myc construct is a potential confounder of the cellular model

Another methodological consideration is the cell type used in the present study. Firstly, the human hippocampal progenitor cell line employed is of foetal origin and as such likely retains youthful molecular and epigenetic signatures (Horvath, 2013), presenting another challenge in extrapolating findings into adult humans, though cells express the same markers as adult hippocampal progenitor cells, and they also have the same potential to proliferate and to differentiate into neurons, astrocytes and oligodendrocytes. Secondly, these cells are transduced with the c-myc-ERTM gene construct (Johansson et al., 2008; Pollock et al., 2006), which could be a potential confounder of the model. This transgene generates a fusion protein that stimulates cell proliferation in the presence of the synthetic drug 4-hydroxy-tamoxifen (4-OHT). Removal of 4-OHT (and the growth factors EGF and FGF) induces differentiation of the hippocampal progenitors into neurons, astrocytes and oligodendrocytes. c-myc is

a highly conserved transcriptional regulatory protein linked to developmental processes and functions as a proto-oncogene in a wide variety of cancers, and its increased expression strongly promotes cell proliferation (Dang et al., 1999). With respect to hippocampal neurogenesis, c-myc is expressed in both the adult dentate gyrus and in cultured adult mouse hippocampal progenitor cells, and is thought to maintain the adult neuronal progenitor cell state. Indeed, c-myc expression is downregulated upon differentiation, and consistent with this, knockdown of c-myc enhances differentiation, whereas overexpression leads to inhibition of differentiation (Denli, 2015). As such, the utilisation of the transgene technology to manipulate its activity enables the recapitulation of key molecular events that regulate adult hippocampal neurogenesis *in vitro*. However, c-myc has been estimated to directly regulate 15-20% of all genes, including those that play a role in cell cycle, cellular growth and proliferation, differentiation and stem cell maintenance, apoptosis, metabolism, ribosome biogenesis leading to elevated protein synthesis, glycolytic activity and oxidative phosphorylation leading to increased energy production and increased level of ROS (Dang, 2012). Importantly, while the overexpression of c-myc has been reported to elevate levels of ROS with concomitant increases in DNA damage (Vafa et al., 2002), a recent study using mice haplo insufficient for c-myc, where its expression was reduced by 50%, reported the same levels of OS markers as in their wild-type aged counterparts (Hofmann et al., 2015), suggesting that c-myc influence on the level of ROS is minimal. Notably, c-myc may also impact upon the immune responses owing to its direct regulation of cytokine expression (Eilers and Eisenman, 2008), however in my experiments cytokines were not detected, neither in vehicle conditions, nor in those treated with *t*-BHP.

Together, studies demonstrate that c-myc activity has a significant impact on the physiological cellular molecular events. As such, the utilisation of the *c-myc-ER*TM construct in the present study may have confounded some of the molecular measures undertaken, such as activation of transcription factors and gene expression. Despite this, the major part of this thesis was devoted to investigation of the effects of redox shifts in neurogenesis (section 4.3) and the changes assessed in these experiments occurred during the differentiation phase, in the absence of 4-OHT, thus limiting the influence of exogenous activation of c-myc on the pathways explored.

4.7.2.2 Immune responses in this cellular model

OS has been shown to trigger inflammation *in vivo* and there are a lot of potential mechanisms by which OS might cause inflammation, as discussed in the introduction of this thesis (section 1.2.6.) Conventionally, hippocampal progenitor cells have been generally considered to be affected by inflammation rather than to produce inflammatory molecules. However, they are known to respond to and produce inflammatory signalling (Horowitz et al., 2015; Johansson et al., 2008; Kokaia et al., 2012; Zunszain et al., 2012a). With respect to immune responses in the brain, glia and in particular microglia have been regarded as the most relevant cell type to investigate. Microglia are known to show pronounced phenotypic changes in response to inflammatory stimuli and to produce inflammatory mediators (Nayak et al., 2014). Additionally, it has been assumed that antidepressants are immunomodulatory largely because of their effects on microglia (Lu et al., 2010; Tynan and Weidenhofer, 2012). However, our lab also demonstrated that monoaminergic antidepressants and omega-3

PUFAs are immunomodulatory directly to hippocampal progenitor cells, extending the scope of cells in which antidepressants can regulate immune processes (Horowitz et al., 2015).

In this study I have shown that hippocampal progenitor cells do not respond to exogenously induced OS by producing pro- or anti-inflammatory cytokines, independently of the OS level being applied. It is possible that this cell line is able to produce immune markers only in response to a very specific stimulus, such as IL-1 β as described above. The absence of inflammatory changes caused by OS in my model could also explain no differences being detected in the kynurenine pathway in cells subjected to oxidative insult, since both OS and inflammation are known to contribute to alterations of this pathway.

4.7.3 Studying OS *in vitro*

Modelling and studying OS in cell culture presents a number of challenges. Cell culture by itself imposes an OS to which cells adapt, changing their properties. In most studies, including those in this thesis, cells are cultured in hyperoxia under atmospheric oxygen concentrations in 1–10 mm Hg range ($\approx 21\%$), whereas under physiological conditions *in vivo* brain oxygen fluctuates around 150 mm Hg ($\approx 5\%$) (Erecińska and Silver, 2001), with O₂ at around 1.3% in the regions with high cellular density such as the hippocampal dentate gyrus. The way to overcome this issue would be culturing cells in special equipment allowing to maintain appropriate O₂ levels, which involves a chamber and a separate glove box set for performing manipulations with cells to avoid changing their exposure to different O₂ levels. As this is an

expensive equipment that is not available in all laboratories (including ours), the majority of published *in vitro* studies have been carried out in standard culturing conditions.

Generally, rates of ROS production increase as O₂ level rises, so cells in culture are also likely to produce more ROS. Thus, it is recognized that cell culture can encourage mutations and genetic drifts, because as soon as cells are placed in culture they begin to adapt and become more resistant to various stimuli, with multiple changes in gene expression and protein levels triggered by the OS. Cellular adaptation to the OS of ‘culture shock’ is characterized by increased ROS production, altered cellular redox, inhibited cell growth, and enhanced antioxidant defences, such as upregulation of superoxide dismutases, GSH synthesis and downregulation of ROS-generating enzymes (Halliwell and Gutteridge, 2015). Therefore, the metabolism of cells in culture is seriously perturbed and cells cultured under atmospheric concentrations of O₂ are preconditioned to tolerate subsequent oxidative insults (Kumar et al., 2016). In addition, cells in culture may even further adapt to use ROS, e.g., for pro-proliferative or anti-death signalling purposes, in ways that might not be relevant *in vivo* (Halliwell, 2003).

Despite the obstacles described above it must be noted that these problems are universal to most of the *in vitro* studies. However, enormous amount of valuable information about metabolic pathways, mechanisms involved in signal transduction, regulation of gene expression, cell proliferation and cell death obtained from cell culture research should not be underappreciated. Also, in this thesis all treatment conditions were always compared to vehicle condition, which existed in the same environment and yet demonstrate significant changes that might be relevant *in vivo*.

4.8 Potential applications of the cellular model and future directions

The cell line utilized in this thesis is a useful *in vitro* model for studying the role of OS in the pathogenesis of depression. It presents a convenient way for identifying specific molecular mechanisms involving redox shifts that could be pharmacologically targeted. It is also a convenient way of screening potential antidepressant compounds for their antioxidative properties. Therefore, following up the data obtained in this thesis, further expanding the exploration of the time course of action of oxidative insults and their effects on DNA and protein damage would be a good supplement to the available data. A huge number of antidepressants from different pharmacological classes available on the market and compounds with potential antidepressant, neuroprotective and antioxidative properties, such as minocycline and erythropoietin, could be tested and further investigated in the model employed in this thesis.

To further study interactions between redox and inflammatory system, it would be interesting to build on the model used in this thesis to introduce more complex interactions and to explore the effect of exogenously-induced OS on microglial inflammatory profile and cytokines secretion. In addition, to the role of microglia in redox regulation, it also plays an important role in the immune cellular environment (Vilhardt et al., 2016). Following this, co-incubation of OS-activated microglia with the hippocampal progenitor cells could be useful. Additionally, a lot of useful information has been obtained from *in vitro* models possessing some of the properties of the blood-brain barrier by co-culturing endothelial cells with brain cells (Abbott et al., 2012; Hurst and Fritz, 1996), which could be also a possibility for studying OS in human hippocampal progenitor cells to investigate what metabolic relationships vascular and neural cell lines might have in OS conditions. Another line of research

could be developed by co-culturing progenitor cells with stem cells. For example, when co-cultured with primary cortical neurons mesenchymal stromal cells were found to protect neurons from damage after experimental stroke and *in vitro* using oxygen–glucose deprivation model of cerebral ischemia (Scheibe et al., 2012).

With regards to the action of EPA, that is known to possess antidepressant properties and has exhibited antioxidative effects in my model, further exploration of the time course of action and specification of the oxidative damage it prevents (lipid, DNA and protein oxidative damage), as well as its ability to modulate transcription factors would be instructive. Additionally, EPA positively modulated system x_c^- that transports one molecule of cystine necessary for GSH synthesis into cells in exchange for one molecule of glutamate into the extracellular space through upregulation of the *SLC7A11* gene. However, no effect of EPA on EAAT3, that clears up excitotoxic glutamate from the extracellular space, was observed. Thus, it would be instructive to investigate a combined effect of EPA and an EAAT3 enhancer in the presence of OS on the glutamate levels to test if such a combination can lead to both increased GSH synthesis and glutamate clearance. In contrast, GSH negatively modulated system x_c^- , potentially leading to decreased extracellular glutamate, which would be interesting to evaluate. Data obtained in this thesis suggest that OS-induced changes in neurogenesis might occur in biological systems with altered redox balance, and a potential ROS-dependent ERK1/2 signal transduction pathway has been identified in the human hippocampal progenitor cells. A next step would be testing and validating the EPA effects on redox-dependant pathways in animal models of depression.

It would be a massive step forward to assess the efficacy of antidepressant drugs in ameliorating a signal indicative of OS in the brain in depressed versus non-depressed subjects using modern neuroimaging techniques, such as positron emission

tomography or magnetic resonance imaging. For example, GSH levels in the brain provide indirect information on oxidative stress of the brain. As such, *in vivo* detection of GSH from various brain regions (frontal cortex, parietal cortex, hippocampus and cerebellum) in bilateral hemispheres of healthy subjects and from bi-lateral frontal cortices in patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD) has been previously reported (Mandal et al., 2012). Similar studies could be conducted with depressed patients.

The purpose of my whole research on the role of OS in MDD was to elucidate specific molecular mechanisms that could be targeted, with a longer term aim to help design more efficient treatments for patients who show only partial response to therapy or who remain treatment-resistant. To further advance this challenge, along with investigating the mechanisms of OS involvement in the pathogenesis of depression, it is crucial to find a reliable method to identify those MDD patients with increased redox status who might benefit from a therapy targeting pathways altered by increased OS. At the moment one of the major challenges in the field of redox biology remains an absence of a standard biomarker or reference levels for OS status *in vivo* and *in vitro*. This brings up a clear necessity to employ a robust OS biomarker, that would detect a major part of the total ongoing oxidative damage *in vivo*.

Findings obtained in this thesis, proposed future research and potential implications are summarized in **Table 11**.

Dimension of research	Results obtained upon <i>t</i> -BHP treatment	Future research and implications
Oxidative damage and its prevention	<ul style="list-style-type: none"> Decrease in cell viability in a dose-dependent manner Increase in lipid peroxidation in a dose-dependent manner Decrease in the number of cells in a dose-dependent manner No difference in the levels of CC3 expression Pre-treatment with GSH, NAC and EPA followed by co-treatment with <i>t</i>-BHP prevented decrease in cell viability No effect for sertraline, venlafaxine or DHA on cell viability in the presence of <i>t</i>-BHP 	<ul style="list-style-type: none"> Investigate different time-points and concentrations Add other OS damage assays, in particular DNA/RNA oxidation and protein carbonyl assays Further identify cell death mechanisms induce by <i>t</i>-BHP (e.g. cytochrome c release, mitochondria membrane potential) Measure intracellular GSH levels and GSH:GSSG ratio upon GSH treatment Use this model as a screening tool for antioxidative properties of other psychotropic medications
Transcription factors	<ul style="list-style-type: none"> Increase in Nrf2 nuclear translocation Increase in NF-κB nuclear translocation 	<ul style="list-style-type: none"> Test the effects of EPA pre-treatment on expression of these transcription factors Identify particular downstream genes using microarray technique
Neuroinflammation	<ul style="list-style-type: none"> No cytokines detected using two different methods (ELISA and Luminex platform) 	<ul style="list-style-type: none"> Test whether Nrf2 mediates inhibition of proinflammatory cytokines independent of ROS elimination Co-culture human hippocampal progenitor cells with microglia
Tryptophan metabolites pathway	<ul style="list-style-type: none"> No difference in tryptophan metabolites 	<ul style="list-style-type: none"> Co-culture human hippocampal progenitor cells with microglia
Signalling pathways	<ul style="list-style-type: none"> Increase in ERK1/2 phosphorylation No difference in JNK and p38 phosphorylation 	<ul style="list-style-type: none"> Investigate different time-points and concentrations of <i>t</i>-BHP for JNK and p38 phosphorylation

Neurogenesis	<ul style="list-style-type: none"> • Decrease in Ki-67 expression, indicative of decreased proliferation • Increase in Dcx and MAP2 expression, indicative of increased neurogenesis • Inhibition of the <i>t</i>-BHP–induced ERK1/2 activation abrogated MAP2 but not Dcx increase 	<ul style="list-style-type: none"> • Co-treatment with antidepressants and PUFAs to assess their effect on neurogenesis in the presence of OS stimulus • Replication of findings <i>in vivo</i>
Gene expression	<p><u><i>t</i>-BHP only:</u></p> <ul style="list-style-type: none"> • Decrease in <i>SLC7A11</i> and <i>Keap1</i> expression • Increase in <i>HMOX1</i> expression • No difference in <i>NQO1</i>, <i>GCLM</i>, <i>SLC1A1</i> expression <p><u>Pre-treatment with GSH or EPA followed by co-treatment with <i>t</i>-BHP:</u></p> <ul style="list-style-type: none"> • EPA and GSH induced further decrease in <i>Keap1</i> expression • EPA induced further increase in <i>HMOX1</i> expression • EPA induced increase (compared with <i>t</i>-BHP treatment only), whereas GSH induced a further decrease in <i>SLC7A11</i> expression • GSH, but not EPA, induced decrease in <i>SLC1A1</i> expression • No difference in <i>NQO1</i> and <i>GCLM</i> expression when pre-treated with GSH or EPA 	<ul style="list-style-type: none"> • Investigate the effect of EPA and GSH on their own (without OS stimulus) on expression of the genes studied in this thesis • Measure GSH and glutamate upon modulation of system x_c^- by EPA and GSH demonstrated in this thesis • Investigate a combined effect of EPA and an EAAT3 enhancer in the presence of OS on the glutamate levels, to test if such a combination can lead to both increased GSH synthesis and glutamate clearance • Measure expression of other OS relevant genes, e.g. genes coding for NOX isoforms, and study the effect of GSH and EPA on their expression in OS conditions

Table 11. Summary of the findings obtained in this thesis, proposed future research and potential implications of the results.

4.9 Conclusion

In this thesis, I have set up for the first time an experimental *in vitro* model of OS in human hippocampal progenitor cells, clinically-relevant to depression. In particular, I have established that *t*-BHP-induced OS caused significant reductions in cell viability and an increase in lipid peroxidation, indicative of oxidative damage, in a dose-dependent manner. This model provided a platform to study potential molecular mechanisms thought to be involved in the pathogenesis of MDD, as well as to test the ability of antidepressant compounds to prevent detrimental changes caused by an oxidative damage.

I have generated evidence on the influence of redox shifts on the human hippocampal neurogenesis, where a mild oxidative stimulus led to an increase in neuroblasts and mature neurons. I have also identified redox-regulated signal transduction pathways involved in these processes. Specifically, I have shown that blocking ERK1/2 activation abrogated the peroxide-induced differentiation of the progenitor cells into mature neurons. The research community is still at the stage of acquiring knowledge on adult neurogenesis and my findings considerably contribute to the field and present a substrate for pharmacological targeting in MDD patients by regulation of redox imbalance in the neurogenic niches.

I have detected OS-induced activation of the Nrf2-Keap1 pathway, which plays a major role in the antioxidant defence, as well as activation of the redox-sensitive transcription factor NF- κ B, a master regulator of immune responses. Contrary to my expectations, NF- κ B activation did not lead to the activation of inflammatory cascades and alterations in the kynurenine pathway, possibly due to the counteraction of Nrf2. Confirmation of this hypothesis by further research could provide a strong reason to believe that therapeutic approaches inducing the Nrf2-Keap1 pathway,

consequently causing an upregulation of antioxidative defences and inhibition of inflammatory responses, might advance existing treatment strategies for MDD.

Finally, I have shown in this thesis for the first time that EPA, known to possess antidepressant properties, prevented oxidative damage in human hippocampal progenitors, in the same way as the conventional antioxidants NAC and GSH, extending findings in animals and peripheral human samples. However, sertraline, venlafaxine, ketamine and DHA, previously reported to modulate the oxidative status in several models, did not demonstrate cytoprotective properties in my model. Moreover, a significant step was taken towards understanding the regulation of gene expression underlying the antioxidative properties of EPA and GSH, which had common as well as differential effects. The revelation of positive modulation of genes involved in the OS response by EPA implies its profound influence on redox status and extends our understanding of reported positive effects of the compound in depression. These data suggest that the oxidative status may identify those patients who might benefit from fish oils consumption to improve depressive symptoms. Remarkably, GSH was identified as a negative regulator of the antioxidative system x_c^- , potentially inhibiting cystine recruitment for further GSH synthesis, which would result in the decrease of extracellular glutamate. Alongside, GSH downregulated EAAT3 expression potentially leading to decreased glutamate clearance from the synaptic cleft, suggestive of a tight balance between GSH synthesis and extracellular glutamate release, both of which have been implicated in the pathophysiology of depression.

In conclusion, findings obtained in this thesis extend our current understanding of redox-related molecular mechanisms considered to underlie the pathophysiology of MDD, that could be potentially pharmacologically targeted, based on the oxidative

status of depressed patients. Also, this research provided further molecular evidence of the beneficial effects of EPA on counterbalancing OS, known to be increased in depressed patients, thus emphasizing that redox state is a defining factor of positive therapeutic response to omega-3 PUFAs supplementation in MDD.

5 References

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